The intracellular calcium antagonist TMB-8 [8-(*NN*-diethylamino)octyl-3,4,5-trimethoxybenzoate] inhibits mitochondrial ATP production in rat thymocytes

Martin D. BRAND and Suzanne M. FELBER

Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

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1. TMB-8 inhibited respiration of rat thymocytes and rat liver mitochondria, probably by inhibition of NADH dehydrogenase.
2. TMB-8 markedly decreased both the cellular ATP concentration and the mitochondrial membrane potential *in situ* in thymocytes.
3. These effects occurred at, or well below, the concentrations used in other systems to investigate the role of intracellular calcium pools in signalling events.
4. We conclude that caution should be exercised in the interpretation of the effects of TMB-8.

There is currently a great deal of interest in the identification and characterization of intracellular stores of calcium and their role in signal transmission and amplification. Inhibitors of the movement and action of calcium are therefore of considerable importance (reviewed by Rahwan, 1983).

One such compound which has been increasingly used is the antiarrhythmic trimethoxybenzoate derivative, TMB-8. Its exact mode of action on cells is not known; however, it is reported to be an 'intracellular calcium antagonist' and is thought to act by stabilizing intracellular membrane-bound calcium or inhibiting calcium transport across some non-mitochondrial subcellular membrane, perhaps the endoplasmic reticulum. The main evidence for this site of action is that TMB-8 blocks contraction in smooth and skeletal muscle in response to agents which cause release of calcium from intracellular stores, and inhibits caffeine-induced calcium release from isolated sarcoplasmic reticulum (Malagody & Chiou, 1974*a,b*; Chiou & Malagody, 1975).

Since these early studies TMB-8 has been used in a number of systems, primarily to support other evidence that suggests the involvement of intracellular calcium pools in various phenomena. The inhibition caused by TMB-8 is not mimicked by extracellular EGTA and is often reversed by

extracellular calcium in the presence of calcium ionophores. This supports the idea that TMB-8 prevents intracellular calcium transport, but we know of no thorough studies of the way in which this compound acts in any of these systems. TMB-8 has been used with platelets (Charo *et al.*, 1976; Le Breton & Dinerstein, 1977; Gorman *et al.*, 1979; Rittenhouse-Simmons & Deykin, 1978; Shaw & Lyons, 1982), fibroblasts (Owen & Villereal, 1982; 1983; Mix *et al.*, 1984), adrenocortical cells (Rubin *et al.*, 1980), neutrophils (Smith & Iden, 1979; Smith *et al.*, 1980; Smolen *et al.*, 1981), other polymorphonuclear leucocytes (Matsumoto *et al.*, 1979; Juhl *et al.*, 1982) and slime moulds (Europe-Finner & Newell, 1984).

We have been investigating membrane potentials and intracellular calcium movements in lymphocytes before and after treatment with mitogens (Dippenaar & Brand, 1982; Felber & Brand, 1982*a,b*, 1983*a,b*; Baumgarten *et al.*, 1983; Brand & Felber, 1984). During this work we used TMB-8 to investigate the participation of non-mitochondrial calcium pools in the mitogenic response. It became clear that TMB-8 had severe side effects on thymocytes. It acted as a potent inhibitor of mitochondrial electron transport, and greatly lowered cellular ATP levels. The present paper reports these effects.

Experimental

Rat thymocytes

These were prepared from 3–5-week-old Wistar rats. The thymus was removed, washed, and placed in culture medium (RPMI 1640 without

Abbreviations used: TMB-8, 8-(*NN*-diethylamino)-octyl-3,4,5-trimethoxybenzoate; TPMP⁺, methyltriphenylphosphonium cation; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazide; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

glutamine, containing 10mM-Hepes and brought to pH7.4 with NaOH). Cells were teased out, washed by centrifugation at 1000g for 5min, resuspended in culture medium at a density of $(4-6) \times 10^7$ cells/ml and kept on ice until use. Viability was >95% by eosin exclusion; yield was $(5-8) \times 10^8$ cells/thymus.

Rat liver mitochondria

These were prepared as described by Chappell & Hansford (1972) in 250mM-sucrose/1mM-EGTA/5mM-Tris/HCL, pH7.2.

ATP assays

Duplicate samples (250 μ l) from an incubation were added to 550 μ l of 3% (w/v) HClO₄ at 0–4°C. After 10min they were neutralized with 300 μ l of 100mM-Mops (4-morpholinepropanesulphonic acid)/400mM-KOH at 0–4°C. Precipitated protein was sedimented by centrifugation and supernatants were assayed for ATP using a luciferin-luciferase assay in a DuPont 760 Luminescence Biometer. ATP standards were subjected to a similar acidification and neutralization procedure.

Oxygen consumption

Rat thymocytes (5×10^7 cells/ml) were suspended in RPMI 1640 culture medium at 37°C for 15min. A sample (2ml) was transferred to a Clark-type oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.) maintained at 37°C containing 2ml of fresh medium. Rat liver mitochondria (0.23mg of protein/ml) were suspended in 120mM-KCl/1mM-EGTA/3mM-Hepes, pH7.0, at 30°C. Glutamate (8mM) plus malate (16mM) was added as substrate. Other additions are described in Fig. 1.

Materials

TMB-8 was from Aldrich; RPMI 1640 culture medium was from Flow Laboratories, Irvine, Scotland, U.K.; luciferase was from Sigma; [³H]TPMP bromide was from New England Nuclear and other radiochemicals were from Amersham International. All other reagents were of the highest grade commercially available.

Results and discussion

Effects of TMB-8 on respiration

Fig. 1(a) shows that 50 μ M-TMB-8 caused a very severe inhibition of respiration by rat thymocytes suspended in a standard RPMI culture medium. TMB-8 is commonly used at 50 μ M in other cells (e.g. Mix *et al.*, 1984); other studies have used it at 500–600 μ M (e.g. Rittenhouse-Simmons & Deykin, 1978; Smolen *et al.*, 1981) or 1–2mM (e.g. Europe-Finner & Newell, 1984). To investigate this inhibition of respiration, TMB-8 was tested on rat liver mitochondria as shown in Figs. 1(b) and 1(c). TMB-8 at 50 μ M inhibited respiration with NAD⁺-linked substrates. With succinate as substrate it did not prevent coupled respiration, nor stimulation of respiration by the uncoupler FCCP. We conclude that the uncoupling effect of TMB-8 on mitochondria is minimal, but that it causes severe inhibition of some step in the respiratory chain before ubiquinone. Since respiration in intact thymocytes probably utilizes the pyruvate produced by glycolysis, the most likely site of action of TMB-8 is the enzyme common to respiration on glutamate plus malate and on pyruvate, namely NADH dehydrogenase. In passing we note that there are similarities in structure between TMB-8 and ubiquinone, one of the substrates of this enzyme.

Fig. 1. Effect of TMB-8 on respiration of rat thymocytes and rat liver mitochondria

For details see the Experimental section. (a), Thymocytes; (b) and (c), liver mitochondria. Additions where shown were 50 μ M-TMB-8, 5 μ M-rotenone, 2mM-succinate (potassium salt), 0.1 μ M-FCCP.

Fig. 2. *Effect of TMB-8 on TPMP⁺ uptake by thymocytes* Thymocyte suspension (5×10^7 cells/ml) was diluted with an equal volume of medium containing either 50 nM [^3H]TPMP bromide (0.5 $\mu\text{Ci}/\text{ml}$) and 3 μM -tetraphenylboron, or 56 μM - [^3H]inulin (0.8 $\mu\text{Ci}/\text{ml}$) or $^3\text{H}_2\text{O}$ (1.5 $\mu\text{Ci}/\text{ml}$). Intracellular and extracellular volumes and TPMP⁺ accumulations were determined in centrifuged pellets by liquid scintillation counting in Beckman Ready-Solv cocktail as described by Felber & Brand (1982a). Where indicated 50 μM -TMB-8, 5 μM -rotenone or 10 μg of oligomycin/ml (final concentrations) were added with the radiochemicals.

Effect of TMB-8 on TPMP⁺ accumulation

TPMP⁺ is a lipophilic cation that is accumulated by cells because of the potential differences across the plasma and mitochondrial membranes. Because of their greater membrane potential most of the accumulation is into the mitochondria. We have used TPMP⁺ to monitor the plasma membrane and mitochondrial membrane potentials in lymphocytes *in situ* (Felber & Brand, 1982a; Brand & Felber, 1984). Fig. 2 shows that TMB-8 caused a considerable decrease in TPMP⁺ uptake by thymocytes, consistent with inhibition of mitochondrial electron transport and mitochondrial depolarization. The effect of rotenone, a well-characterized inhibitor of NADH dehydrogenase, was similar to that of TMB-8, but not as great. With either TMB-8 or rotenone present the residual TPMP⁺ accumulation was larger than that predicted for the plasma membrane alone, which, with a membrane potential of about 60 mV, should give about 10-fold accumulation of TPMP⁺ (Felber & Brand, 1982a). Fig. 2 shows that addition of oligomycin plus rotenone did reduce TPMP⁺ accumulation a little further. Oligomycin inhibits the mitochondrial H^+ -ATPase, so should prevent energization of the mitochondria by ATP derived from glycolysis. We conclude that TMB-8 causes a substantial collapse of the mitochondrial membrane potential (which will lead to calcium efflux by reversal of the uniporter; see Nicholls & Åkerman, 1982), with a

Fig. 3. *Effect of TMB-8 on thymocyte ATP concentration* Thymocytes (5.4×10^7 cells/ml) were preincubated at 37°C in RPMI 1640 culture medium for 20 min, then additions as shown were made. At the times indicated samples (250 μl) were withdrawn and assayed for ATP as described in the Experimental section. ATP concentration was calculated using the measured intracellular volume of 120 fl/cell.

residual potential being maintained, at least partly, by consumption of cytosolic ATP.

Effect of TMB-8 on cellular ATP levels

Fig. 3 shows that 50 μM -TMB-8 caused total thymocyte ATP to drop considerably within 5 min of addition. By 30 min the intracellular ATP concentration was only 25% of the control value. Rotenone caused a slightly greater drop in ATP concentration, with a similar timecourse. TMB-8 at 10 μM had little effect on cell ATP content.

Conclusions

The results we present here show that in rat thymocytes TMB-8 causes severe inhibition of electron flow, mitochondrial depolarization with possible calcium release, and a considerable fall in cellular ATP concentration. It has these effects at concentrations similar to, or well below, those used in other systems. Clearly great care in interpretation must be taken when using this inhibitor in thymocytes. Nonetheless, some of our results (S. M. Felber & M. D. Brand, unpublished work) are consistent with inhibition by TMB-8 of calcium release from intracellular stores, in addition to the effects on mitochondria that are reported here.

Most of the published reports of the action of TMB-8 on other cell types (see the introduction) make no mention of controls for secondary effects on the cells, and so should be treated with caution. In any case we suggest that future studies of intracellular calcium pools using TMB-8 in any system should be carried out sufficiently thoroughly to eliminate the possibility of side effects of this drug.

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