Calcium-independent effects of TMB-8

Modification of phospholipid metabolism in neuroblastoma cells by inhibition of choline uptake

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TMB-8 [8-(*NN*-diethylamino)-octyl-3,4,5-trimethoxybenzoate] blocks agonist-stimulated release of Ca^{2+} from intracellular sites in many cell lines and is often used to distinguish between dependence on extracellular and intracellular Ca^{2+} . In N1E-115 neuroblastoma cells, TMB-8 did not alter the resting cytosolic Ca^{2+} concentration in unstimulated cells, yet phospholipid metabolism was greatly affected. At concentrations of TMB-8 (25–150 μM) that inhibit Ca^{2+} release, phosphatidylcholine formation was inhibited, whereas synthesis of phosphatidylinositol, phosphatidylglycerol and phosphatidylserine was stimulated. Unlike other cationic amphipathic compounds, TMB-8 did not inhibit phosphatidate phosphatase or enzymes in the pathway from choline to phosphatidylcholine. Choline transport was the major site of action. TMB-8 was a competitive inhibitor ($K_i = 10 \mu\text{M}$) of low-affinity ($K_t = 20 \mu\text{M}$) choline transport. When added at the same time as labelled precursor, TMB-8 also decreased cellular uptake of phosphate and inositol, but not that of ethanolamine or serine. In prelabelled cells, continued uptake and incorporation of phosphate and inositol were not affected. Under these conditions phosphatidylinositol synthesis was increased 2-fold and, like the effect on phosphatidylcholine, reached a plateau at 100 μM -TMB-8. Phosphatidylglycerol synthesis increased linearly with TMB-8 concentration to 40-fold stimulation at 150 μM , suggesting a selective effect on synthesis of phosphatidylglycerol from CDP-diacylglycerol. Phosphatidylserine synthesis was also increased up to 3-fold. These Ca^{2+} -independent effects limit the use of TMB-8 in studies of cell signalling that involve stimulated phosphatidylinositol and phosphatidylcholine metabolism.

INTRODUCTION

On the basis of initial studies with muscle, TMB-8 [8-(*NN*-diethylamino)-octyl-3,4,5-trimethoxybenzoate] has been used in many tissues as an intracellular Ca^{2+} antagonist. It is thought to decrease intracellular Ca^{2+} availability by inhibiting release of Ca^{2+} from the sarcoplasmic reticulum [1,2] and stabilizing Ca^{2+} bound to the intracellular surface of t-tubular membranes [3]. In many cells TMB-8 blocks efflux of Ca^{2+} from intracellular stores without affecting influx of extracellular Ca^{2+} , and has often been used to demonstrate dependence on intracellular Ca^{2+} release and to differentiate between dependence on intracellular and extracellular Ca^{2+} .

At concentrations used to study Ca^{2+} -dependent effects, TMB-8 has additional effects on some cells which cannot be attributed to altered Ca^{2+} status. TMB-8 is a competitive inhibitor of muscarinic-receptor binding [4,5] and inhibits carbachol-induced increases in cytosolic Ca^{2+} , PtdIns hydrolysis and enzyme secretion by this mechanism in pancreatic acinar cells [6]. TMB-8 does not inhibit thrombin-induced mobilization of Ca^{2+} and consequent secretion in human platelets, but does block phorbol-ester-induced secretion that neither causes nor requires an increase in cytosolic Ca^{2+} [7]. Phorbol ester activation of superoxide production in human polymorphonuclear leucocytes is also inhibited by TMB-8 by a mechanism that neither affects intracellular Ca^{2+} nor involves direct inhibition of protein kinase C [8]. TMB-8 may also interfere with oxidative metabolism [9], and is antiketogenic by inhibition of mitochondrial carnitine palmitoyltransferase [10]. At high concentrations [0.5–1 mM], TMB-8 is cytotoxic [6], decreases intracellular ATP [11], and

disrupts membrane structure, causing leakage of cytoplasmic constituents [12].

In studies of the role of intracellular Ca^{2+} release on phosphoinositide metabolism, we noted a dose-dependent reciprocal effect on the turnover of PtdIns and phosphatidylcholine (PtdCho) in unstimulated N1E-115 neuroblastoma cells by relatively low concentrations of TMB-8 (10–100 μM). In the present study we determined that this apparently Ca^{2+} -independent action of TMB-8 was due mainly to competitive inhibition of choline uptake into the cells. Possible specific stimulation of PtdIns, 1-phosphatidylglycerol (PtdGro) and phosphatidylserine (PtdSer) synthesis was also observed.

MATERIALS AND METHODS

Materials

Carrier-free $\text{H}_3^{32}\text{PO}_4$, [methyl- ^3H]choline chloride (80 Ci/mmol), [1,2- ^{14}C]ethanolamine hydrochloride (4.3 mCi/mmol), [G- ^3H]serine (14.4 Ci/mmol) and sodium [9,10- ^3H]oleate (10.0 Ci/mmol) were obtained from DuPont Canada (NEN Products, Lachine, PQ, Canada). *myo*-[2- ^3H]inositol (16.2 Ci/mmol) was from Amersham (Oakville, Ont., Canada). Phospholipids used as chromatographic standards were from Serdary Research Laboratories (London, Ont., Canada). Fura-2 AM (stored as a 1 mM stock solution in dimethylsulphoxide at -20°C), TMB-8 and chlorpromazine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Lipid labelling experiments

Mouse neuroblastoma cells (N1E-115) were maintained in

Abbreviations used: PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; PtdOH, phosphatic acid; PtdGro, 1-phosphatidylglycerol; (Ptd) $_2$ Gro, 1,3-bisphosphatidylglycerol; TMB-8, 8-(*NN*-diethylamino)-octyl-3,4,5-trimethoxybenzoate; DMEM, Dulbecco's modified Eagle's medium.

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150 cm² plastic flasks in 45 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) fetal-calf serum and 5% (v/v) newborn-calf serum as described previously [13]. For all incorporation studies, $(0.5\text{--}1.0) \times 10^6$ cells were transferred to 60 mm-diam. plastic Petri dishes in 6.5 ml of culture medium. After 72 h the medium was removed and replaced with 2.0 ml of DMEM (without serum). For pulse-label experiments, radiolabelled precursors (10 μCi of [³²P]P_i, 2 μCi of [2-³H]inositol, 4 μCi of [G-³H]serine, 2 μCi of [methyl-³H]choline or 2 μCi of [1,2-¹⁴C]ethanolamine) were suspended in DMEM and added to cultures with and without TMB-8 (in 20 μl of dimethyl sulphoxide). After 1 h (20 min for measurement of choline transport into the cells), medium was removed and cells were washed twice with 2 ml of ice-cold phosphate-buffered saline (137 mM-NaCl/2.7 mM-KCl/0.5 mM-MgCl₂/1.5 mM-KH₂PO₄/8.0 mM-Na₂HPO₄, pH 7.0). For continued pulse experiments, cells were prelabelled by incubating for 1 h with the radiolabelled precursor before addition of TMB-8. In experiments with [9,10-³H]oleate, radioactive oleate and carrier oleate were dried together under nitrogen and suspended in sterile 5% BSA (fatty acid/BSA ratio 5), and 2 μCi was added to each dish. After an additional 30 min incubation, the medium was removed and the cells washed.

Water-soluble and lipid metabolites were extracted from washed cells as described previously [14]. Protein was determined by the method of Lowry *et al.* [15]. Phospholipids were resolved by unidirectional t.l.c. on silica gel G plates (Fisher Scientific, Dartmouth, N.S., Canada) or silica gel HR plates (Analtech, Newark, NJ, U.S.A.) developed with chloroform/ethanol/water/triethylamine (4:5:1:4, by vol.) [16]. Unlabelled marker phospholipids were added to each sample to facilitate detection. For two-dimensional t.l.c., plates were developed first with chloroform/methanol/water/14.8 M-NH₃ (48:40:7:5, by vol.), dried thoroughly and developed in the second dimension with chloroform/methanol/formic acid (11:5:1, by vol.) [17]. Phospholipids were detected with phosphomolybdate spray or iodine vapour, and radioactivity was determined by liquid-scintillation counting of gel scraped from the plate. In experiments with [1-¹⁴C]oleate, neutral lipids were resolved by t.l.c. on silica gel G developed with hexane/diethyl ether/acetic acid (85:15:1, by vol.) [16]. Distribution of radioactivity was determined with a Bioscan Imaging Scanner System 200-IBM (Bioscan Inc., Washington, DC, U.S.A.).

Choline-containing metabolites in the water-soluble upper phases from some experiments with [methyl-³H]choline were analysed by h.p.l.c. on normal-phase silica (Pecosphere, 5 μm particle size, 4.6 mm internal diam. $\times$ 83 mm; Perkin-Elmer, Norwalk, CT, U.S.A.) at 45 °C and a flow rate of 1.5 ml/min using the solvent system of Liscovitch *et al.* [18]. Samples were applied in solvent A (acetonitrile/ethanol/water/ammonium acetate buffer, 800:68:127:5, by vol.). Acetate buffer consisted of acetic acid/1 M-ammonium acetate (4:5, by vol.). A linear gradient from 0 to 10% solvent B (acetonitrile/ethanol/water/ammonium acetate buffer, 100:17:100:33, by vol.) was applied between 3 and 15 min, followed by a linear increase to 100% solvent B at 27 min. Radioactive compounds eluted from the column were measured with a Flow-One\Beta radioactivity flow detector (Radiomatic Instruments and Chemical Co., Tampa, FL, U.S.A.) and identified by comparison of their elution times with those of standards (acetylcholine, 10.5 min; choline, 12.5 min; CDP-choline, 25 min; phosphocholine, 28 min).

Phosphatidate (PtdOH) phosphatase determination on subcellular fractions

Confluent cells (30–40 mg of protein per 150 cm² flask) were washed twice with iso-osmotic saline, suspended in 5 ml of

0.25 M-sucrose containing 20 mM-Hepes (pH 7.4), 0.2 mM-dithioerythritol and 2 mM-EDTA and disrupted by sonication (Branson Sonifier, three 10 s bursts at 60% power). The post-mitochondrial supernatant, obtained by centrifuging the homogenate at 18000 *g* for 10 min (Beckman LH 70 ultracentrifuge), was centrifuged at 105000 *g* for 45 min to yield cytosol and membrane fractions. The membrane pellet was resuspended in the buffered sucrose. Homogenates and subcellular fractions could be stored at –20 °C in 20% (v/v) glycerol for several days without appreciable loss of PtdOH phosphatase activity.

Mg²⁺-dependent PtdOH phosphatase was measured under conditions adapted from Martin *et al.* [19], in which EGTA and EDTA were used to remove polyvalent cations (particularly Ca²⁺) and to avoid any Mg²⁺-independent activity or phospholipase C. Substrate was presented as a mixed emulsion with PtdCho suspended in 2.5 mM-EDTA/2.5 mM-EGTA (pH 7.4). The final optimized reaction mixture (0.15 ml) contained 10 mM-MgCl₂, 1 mM-EDTA, 1 mM-EGTA, 1 mM-dithioerythritol, 2 mg of BSA, 0.9 mM-PtdOH, 0.4 mM egg-yolk PtdCho and 40–50 μg of protein in 50 mM-Hepes buffer (pH 7.4). All components of the reaction mixture except the enzyme were preincubated at 37 °C for 5 min, and the reactions were started by adding 30 μl of the cell fraction to be assayed. After 30 min at 37 °C, reaction mixtures were solubilized by adding 0.35 ml of acidified (pH 4.0) 5% SDS containing 40 mM-EDTA, and P_i was determined by an automated system as described previously [20].

Measurement of intracellular Ca²⁺

Near-confluent monolayers of N1E-115 cells were dislodged into fresh Hepes–DMEM (DMEM buffered with 50 mM-Hepes at pH 7.4, Methyl Red indicator omitted) by tapping the flask, harvested by brief centrifugation and suspended in Hepes–DMEM containing 4 μM Fura-2 AM [$(1\text{--}5) \times 10^6$ cells/ml]. After 1 h at 37 °C, fura-2 loaded cells were washed twice with fresh Hepes–DMEM and suspended in the same medium (approx. 10⁶ cells/ml). Changes in cytosolic free Ca²⁺ were monitored by recording changes in the fluorescence ratio at 510 nm, with excitation at 340 nm/380 nm, in a Perkin-Elmer LS-50 spectrofluorimeter at 37 °C [21].

RESULTS

Effects of TMB-8 on cellular uptake of phospholipid precursors

Pulse-labelling experiments were used to assess effects of TMB-8 on the entry of phospholipid precursors into cells (as measured by appearance of radioactivity in the intracellular pool of water-soluble metabolites) and on the synthesis of phospholipids from those metabolites (Table 1). In experiments with [³²P]P_i, $4.4 \pm 0.1\%$ (mean $\pm$ S.E.M., $n = 12$) of the radioactivity was recovered in the cells (1.59 ± 0.04 mg of protein/dish) after 1 h. Only $1.2 \pm 0.1\%$ of incorporated ³²P was found in the phospholipids. TMB-8 (100 μM) decreased incorporation of [³²P]P_i into the water-soluble compartment (mainly P_i and ATP) by 20% without affecting incorporation into total phospholipid (Table 1), suggesting that synthesis of at least some phospholipids was stimulated by TMB-8. This interpretation was confirmed by t.l.c. separation of individual phospholipids. TMB-8 decreased labelling of phosphatidylethanolamine (PtdEtn) and sphingomyelin to the same extent as for the water-soluble precursors. Incorporation into acidic phospholipids was selectively increased. PtdIns and PtdSer labelling was increased marginally in TMB-8-treated cells, whereas incorporation into PtdGro and 1,3-bisphosphatidylglycerol [(Ptd)₂Gro] was increased by 165%. In contrast, radioactivity appearing in PtdCho was decreased to a greater extent than that in the precursor pool, suggesting that TMB-8 specifically inhibited synthesis of this phospholipid.

Table 1. Effects of TMB-8 on the uptake and incorporation of phospholipid precursors in N1E-115 neuroblastoma cells

N1E-115 cells were grown to near confluency and the medium was replaced with 2.0 ml of fresh medium at the start of the experiment. Radiolabelled precursors (10 μ Ci of [32 P]P_i, 2 μ Ci of [2- 3 H]inositol, 4 μ Ci of [G- 3 H]serine, 2 μ Ci of [methyl- 3 H]choline, or 2 μ Ci of [1,2- 14 C]ethanolamine) were added with and without 100 μ M-TMB-8. After 1 h, cells were harvested and extracted as described in the Materials and methods section. Radioactivity was measured in water-soluble intracellular precursors and the organic phase containing phospholipids. Results are expressed as percentages of control values without TMB-8 (means $\pm$ s.d. for 3–4 experiments).

Precursor	Incorporation (% of control)	
	Water-soluble	Lipid-soluble
[32 P]P _i	80.0 $\pm$ 12.9	103.9 $\pm$ 11.2
[2- 3 H]Inositol	63.5 $\pm$ 11.5	97.0 $\pm$ 10.1
[methyl- 3 H]Choline	26.9 $\pm$ 0.8	28.9 $\pm$ 2.2
[1,2- 14 C]Ethanolamine	114.4 $\pm$ 21.4	112.8 $\pm$ 10.9
[G- 3 H]Serine	101.1 $\pm$ 4.0	110.6 $\pm$ 9.8

In similar experiments with [3 H]inositol, 1.9 ± 0.1 % (mean $\pm$ s.e.m., $n = 8$) of radiolabelled precursor was taken up by control cells from the medium. PtdIns represented 50 ± 1 % of total radioactivity in the cells. TMB-8 (100 μ M) decreased total uptake of labelled precursor into cells to a similar extent as observed for [32 P]P_i (21 %). Incorporation of [3 H]inositol into the water-soluble compartment (almost entirely free inositol; [22]) was decreased by 35 % without affecting appearance of radioactivity in phospholipid (Table 1). The effect of TMB-8 was dose-dependent. At lower concentrations, [3 H]inositol uptake was less affected and incorporation into PtdIns was slightly stimulated (results not shown). At 200 μ M-TMB-8, [3 H]inositol uptake was decreased by 60 %, sufficient to decrease PtdIns labelling by 15–20 % compared with untreated cells.

TMB-8 greatly decreased [3 H]choline uptake into water-soluble precursors of PtdCho, and labelling of PtdCho was decreased proportionately (Table 1). The effect was dose-dependent, but labelling of the precursor pool did not fall below 20 % at concentrations of TMB-8 up to 400 μ M. However, PtdCho labelling was virtually abolished at 150 μ M-TMB-8. Uptake of [G- 3 H]serine into cells and incorporation into phospholipid was very low. Of the 2.3–3.3 % of radioactivity taken up by cells after 1 h, only 3.8 ± 0.2 % ($n = 29$) appeared in PtdSer and 1.0 ± 0.03 % in PtdEtn. Uptake of [1,2- 14 C]ethanolamine into cells was also low (2.0 ± 0.1 % after 1 h; $n = 29$), but a substantial proportion (55.2 ± 1.9 %) appeared in phospholipid. TMB-8 (100 μ M) did not affect either uptake of [G- 3 H]serine and [1,2- 14 C]ethanolamine or their incorporation into phospholipid. PtdCho was not labelled from either [G- 3 H]serine or [1,2- 14 C]ethanolamine, confirming the absence of PtdEtn methylation in these cells.

Effects of TMB-8 on phospholipid synthesis

To decrease the complication of altered precursor uptake into cells, precursor pools were prelabelled for 1 h. Effects on phospholipid metabolism were then monitored after a short additional incubation (30 min) in the presence and absence of various concentrations of TMB-8 (continued pulse). Incorporation of [32 P]P_i into water-soluble components increased by 25 % during the second pulse phase, but this was not changed by up to 100 μ M-TMB-8 (Fig. 1). With the effect on [32 P]P_i uptake into the phospholipid precursor pool decreased, specific effects on phospholipid metabolism were more clearly defined. Labelling of all phospholipids increased by about 75 % during the second pulse phase in control cells. PtdCho synthesis during the second pulse phase was decreased in a dose-dependent fashion and effectively stopped by 150 μ M-TMB-8. Labelling of PtdEtn, synthesized by a similar pathway, was decreased by less than 10 %. Synthesis of all acidic phospholipids [PtdIns, PtdGro and (Ptd)₂Gro] was stimulated by TMB-8, although the concentration-dependence was different for each phospholipid. Radioactivity in PtdIns reached a maximum 2.3-fold increase at 75–100 μ M-TMB-8. Radioactivity in PtdGro + (Ptd)₂Gro continued to increase to 8–10-fold at 150 μ M-TMB-8. In contrast, PtdSer labelling was relatively unaffected below 75 μ M-TMB-8, but was increased 3-fold at 150 μ M.

Acidic phospholipids were not resolved by one-dimensional t.l.c. [PtdOH co-migrated with PtdIns and PtdGro with (Ptd)₂Gro]. Two-dimensional analysis of lipid extracts from cells exposed to 150 μ M-TMB-8 during the second pulse phase with [32 P]P_i (Table 2) showed that PtdOH was turning over rapidly, as indicated by the substantial amount of radioactivity found in control cells at 1 h. Although values were variable, labelling of PtdOH declined during the second pulse phase in control cells, but was maintained in TMB-8-treated cells. The TMB-8-dependent increase in the PtdIns/PtdOH band from one-dimensional chromatography was due to increased labelling of

Fig. 1. Effects of TMB-8 on phospholipid metabolism after prelabelling of precursor pools with [32 P]P_i

N1E-115 cells were grown as described in the text. [32 P]P_i (2 μ Ci/dish) was added and the cells were incubated for 1 h. TMB-8 in 20 μ l of dimethyl sulphoxide, or dimethyl sulphoxide alone (controls), was added and the incubation continued for 30 min. Aqueous and lipid extracts were prepared and phospholipids were analysed by one-dimensional t.l.c. Radioactivity in each phospholipid and the aqueous extract at the end of the 90 min incubation is expressed as a percentage of the radioactivity in the corresponding component at the end of the 60 min prelabelling period. Data are means $\pm$ s.d. for four experiments. The upper panel shows water-soluble components (○), PtdIns (●), PtdCho (▲), and PtdEtn (■). PtdSer (□) and PtdGro + (Ptd)₂Gro (■) are in the lower panel.

Table 2. Effects of TMB-8 on phospholipid turnover in N1E-115 cells

N1E-115 cells were prelabelled with [32 P]P_i (2 μ Ci/dish) for 1 h, and the incubation was continued for 30 min in the absence (control) and presence of 150 μ M-TMB-8. Aqueous and lipid extracts were prepared and phospholipids analysed by two-dimensional t.l.c. Data are means $\pm$ s.d. of triplicate dishes from one of two similar experiments.

	10 ⁻³ $\times$ Radioactivity (d.p.m./min per mg of protein)		
	Control 60 min	Control 90 min	TMB-8 90 min
Aqueous phase	2310 $\pm$ 313	2932 $\pm$ 179	2692 $\pm$ 104
PtdOH	3.3 $\pm$ 0.8	1.6 $\pm$ 0.4	3.7 $\pm$ 0.6
CMP-PtdOH	0.7 $\pm$ 0.02	1.9 $\pm$ 0.1	4.8 $\pm$ 0.1
PtdGro	2.0 $\pm$ 0.2	2.3 $\pm$ 0.9	14.1 $\pm$ 0.5
(Ptd) ₂ Gro	1.1 $\pm$ 0.3	1.7 $\pm$ 0.1	5.1 $\pm$ 0.6
PtdIns	15.0 $\pm$ 1.7	23.3 $\pm$ 3.5	39.8 $\pm$ 3.4
PtdCho	9.2 $\pm$ 1.1	16.4 $\pm$ 0.8	10.6 $\pm$ 1.2
Sphingomyelin	0.5 $\pm$ 0.01	0.5 $\pm$ 0.1	0.5 $\pm$ 0.1
PtdEtn	10.9 $\pm$ 1.7	16.7 $\pm$ 2.3	14.0 $\pm$ 0.8
PtdSer	0.6 $\pm$ 0.04	1.1 $\pm$ 0.4	1.6 $\pm$ 0.1
Protein (mg/dish)	2.8 $\pm$ 0.3	2.9 $\pm$ 0.3	3.1 $\pm$ 0.1

PtdIns. Labelling of CMP-PtdOH, also a quantitatively minor but metabolically active intermediate, was stimulated 3.4-fold by TMB-8. The substantial TMB-8 stimulation of the PtdGro + (Ptd)₂Gro band on one-dimensional t.l.c. plates was due mainly to PtdGro. Although PtdGro is normally present only in trace amounts in N1E-115 cells, labelling of this phospholipid was increased 35–45-fold by TMB-8. In contrast, (Ptd)₂Gro, which represents approx. 6% of the phospholipid mass [22], was labelled to a lesser extent and stimulated only 7-fold by TMB-8. PtdEtn labelling was decreased by TMB-8. This result was not apparent in other experiments (Fig. 1), was not concentration-dependent (observed only at the highest concentration of TMB-8) and was proportionate to the decreased labelling of the water-soluble precursor pool, which also occurred only at high TMB-8 concentration. Thus decreased PtdEtn labelling in this experiment was more likely related to decreased entry of [32 P]P_i into the precursor pool than to a specific effect of TMB-8 on PtdEtn metabolism.

TMB-8 did not affect continued uptake of [14 C]ethanolamine by N1E-115 cells, but decreased the amount of radioactivity appearing in PtdEtn (Fig. 2a). In this case the decrease was concentration-dependent. Total radioactivity appearing in the cells from [3 H]inositol was not altered by TMB-8 during the second pulse period (Fig. 2b). Increased radioactivity in PtdIns was at the expense of a corresponding decrease in radioactive inositol in the aqueous compartment (equal amounts of radioactivity were present in the two compartments in control cells). Maximum stimulation of PtdIns labelling was similar to that seen in experiments with [32 P]P_i (2-fold), but less than observed for the other acidic phospholipids derived from the same precursors (PtdOH and CMP-PtdOH). Since labelled intracellular inositol was not exhausted, decreased precursor availability does not seem to restrict TMB-8-stimulated PtdIns synthesis. TMB-8 did not affect the amount of radioactivity from [3 H]serine appearing in the aqueous compartment during the second pulse phase (Fig. 2c), but did cause a concentration-dependent increase in labelling of PtdSer. Analysis of isolated PtdSer by phospholipase C cleavage [23] showed that 96 $\pm$ 3% of the radioactivity was in the serine moiety. Labelling of PtdEtn

Fig. 2. Modification by TMB-8 of phospholipid synthesis from ethanolamine, inositol and serine

Experimental procedures were as described in Fig. 1, except that cells were prelabelled with [1,2- 14 C]ethanolamine (a), [2 - 3 H]inositol (b) or [G- 3 H]serine (c) before addition of TMB-8. Data are means $\pm$ s.d. (four experiments) for water-soluble components (○) (all panels), PtdEtn (●), PtdIns (■) and PtdSer (▲).

(presumably formed by decarboxylation of PtdSer) was also increased to the same extent.

Even in continued pulse experiments, TMB-8 (> 100 μ M) decreased the continued entry of [*methyl*- 3 H]choline into the water-soluble compartment of cells by 70% (Fig. 3). Total radioactivity in water-soluble metabolites (including prelabelled compounds) was decreased by only 33% by TMB-8. Nevertheless, TMB-8 decreased PtdCho labelling by 60% in the second pulse phase, suggesting that prelabelled metabolites were not available for PtdCho synthesis.

TMB-8 does not inhibit PtdOH phosphatase

Some cationic amphipathic compounds that alter phospholipid metabolism inhibit PtdOH phosphatase [24]. Since triacylglycerol synthesis *de novo* is dependent on availability of diacylglycerol from PtdOH, incorporation of labelled oleate into acylglycerols and phospholipids was monitored in the presence of TMB-8 (Table 3). Chlorpromazine, an inhibitor of PtdOH phosphatase [25,26], was used as a positive control. Cells were preincubated for 1 h to label intracellular precursor pools and emphasize effects occurring later in the metabolic pathways. Incubation was continued for 30 min after addition of TMB-8 or chlorpromazine. Incorporation of [9,10- 3 H]oleate was linear for

Fig. 3. Inhibition of PtdCho synthesis by TMB-8

Experimental procedures were as described in Fig. 1, except that cells were prelabelled with [methyl-³H]choline before addition of TMB-8. Data are means (two experiments) for water-soluble components (○) and PtdCho (▲).

Table 3. Effects of TMB-8 on oleate incorporation into neutral lipids and phospholipids of N1E-115 cells

Cells were grown to near confluence (2.4 ± 0.3 mg of protein/dish; mean $\pm$ s.d., $n = 12$) and transferred to 2 ml of fresh medium containing $100 \mu\text{M}$ -[³H]oleate ($0.04 \mu\text{Ci}/\mu\text{mol}$) prepared in sterile 5% BSA and preincubated for 60 min. After addition of $100 \mu\text{M}$ -TMB-8 or $100 \mu\text{M}$ -chlorpromazine, incubation was continued for 30 min. Lipids were extracted and separated by t.l.c. Oleate incorporation, expressed as nmol/mg of protein, was calculated from the specific radioactivity of the [³H]oleate added to the medium. Data are the means $\pm$ s.d. for three dishes. Differences between values for treated cells and the corresponding 90 min controls were assessed by unpaired Student's *t* test: * $P > 0.1$, ** $0.01 < P < 0.025$, *** $P < 0.005$.

Treatment	Time (min)	³ [H]Oleate incorporation		
		Phospho-lipid	Triacyl-glycerol	Alkyldiacyl-glycerol
Control	60	14.5 $\pm$ 2.7	8.0 $\pm$ 1.9	1.2 $\pm$ 0.3
Control	90	20.8 $\pm$ 1.3	11.8 $\pm$ 1.6	2.2 $\pm$ 0.3
TMB-8	90	20.8 $\pm$ 0.9*	12.7 $\pm$ 0.6*	2.3 $\pm$ 0.3*
Chlorpro-mazine	90	22.9 $\pm$ 3.0*	7.9 $\pm$ 0.4***	1.5 $\pm$ 0.3**

the duration of the experiment, with 17.5% of available radioactivity appearing in cellular lipids of untreated cells after 90 min. Although both compounds alter synthesis of individual phospholipids, neither TMB-8 nor chlorpromazine changed labelling of the total phospholipid pool with oleate in the last 30 min of incubation. No radioactivity co-chromatographed with diacylglycerols in lipid extracts from control or treated cells. Thus diacylglycerol did not accumulate in these cells, and triacylglycerol synthesis would be particularly dependent on PtdOH phosphatase activity. Consistent with its ability to inhibit PtdOH phosphatase, chlorpromazine blocked synthesis of triacylglycerols. However, TMB-8 had no effect on triacylglycerol synthesis. A major portion (15–20%) of the radioactivity appearing in neutral lipid co-chromatographed with alkyldiacylglycerols. Chlorpromazine, but not TMB-8, blocked labelling of this component as well.

As previously reported for rat liver [19] and lung [27], chlorpromazine inhibited PtdOH phosphatase activity by 55% when added to homogenates of N1E-115 cells in an assay mixture *in vitro* at concentrations exceeding the concentration of substrate (1.5 mM-chlorpromazine/1 mM-PtdOH). In contrast,

Fig. 4. Competitive inhibition of choline transport into N1E-115 cells by TMB-8

Cells were incubated with increasing concentrations of [³H]choline for 20 min at 37 °C without TMB-8 (○), with $50 \mu\text{M}$ -TMB-8 (▲) or with $75 \mu\text{M}$ -TMB-8 (■). The non-saturable diffusion component of choline uptake (○) was determined by incubating cells at 37 °C with an excess of unlabelled choline ($50 \mu\text{M}$) or at 0–4 °C (see the text). Radioactivity was measured in the water-soluble compounds (choline + phosphocholine), and the amount of choline taken up by the cells was calculated from the specific radioactivity of [³H]choline in the medium. Data in the left panel are means $\pm$ s.e.m. for 16 dishes from 8 separate experiments for cells incubated at 37 °C without TMB-8, and means $\pm$ s.d. for 4 dishes from two experiments for all others. The right-hand panel shows a double-reciprocal plot of the means of the same data after subtraction of the diffusion component (units as in the left-hand panel). Straight lines were calculated by linear-regression analysis.

Table 4. Effects of TMB-8 and chlorpromazine on the incorporation of [³H]choline into N1E-115 cells

Cells were grown to near confluence (1.9 ± 0.1 mg of protein/dish for 20 min experiments, 2.1 ± 0.1 mg of protein/dish for 60 min experiments; means $\pm$ s.d.) and transferred to 2 ml of fresh DMEM containing $28 \mu\text{M}$ -choline. After addition of $75 \mu\text{M}$ -TMB-8 or $75 \mu\text{M}$ -chlorpromazine, $4.5 \mu\text{Ci}$ of [methyl-³H]choline was added to each dish and the cells were incubated for 20 or 60 min. Water-soluble metabolites were analysed by h.p.l.c., and PtdCho was isolated by t.l.c. Choline incorporation (pmol/mg of protein) was calculated from the specific radioactivity of [³H]choline present in the medium. Data are means $\pm$ s.d. with numbers of dishes analysed in parentheses.

	Total incorporation (pmol/mg of protein)		
	Control	TMB-8	Chlorpromazine
20 min	1772 $\pm$ 335 (6)	380 $\pm$ 163 (6)	1044 $\pm$ 228 (6)
60 min	3312 $\pm$ 929 (9)	744 $\pm$ 173 (8)	1259 $\pm$ 109 (6)
	Distribution (% of [³ H]choline incorporated)		
	Control	TMB-8	Chlorpromazine
20 min			
Choline	71 $\pm$ 5	74 $\pm$ 10	72 $\pm$ 5
Phosphocholine	29 $\pm$ 5	26 $\pm$ 10	28 $\pm$ 5
PtdCho	0.4 $\pm$ 0.1	0.2 $\pm$ 0.1	0.1 $\pm$ 0.1
60 min			
Choline	57 $\pm$ 13	64 $\pm$ 15	76 $\pm$ 10*
Phosphocholine	40 $\pm$ 11	34 $\pm$ 14	21 $\pm$ 12*
PtdCho	3.6 $\pm$ 2.7	1.8 $\pm$ 1.7	2.4 $\pm$ 2.3

* Significant difference from control ($P < 0.005$, unpaired *t* test).

TMB-8 had no effect at the same concentration. PtdOH phosphatase is an ambiquitous enzyme with membrane-bound activity believed to be the active form *in vivo* [25,26,28]. In the post-mitochondrial fraction of N1E-115 cells $26 \pm 1.7\%$ of the activity was associated with membranes. This distribution was not altered by adding TMB-8 ($100 \mu\text{M}$) directly to the post-mitochondrial supernatant or by pretreating whole cells for 10 min before fractionation. Fatty acid causes translocation of PtdOH phosphatase from the cytosol to intracellular membranes [19,29,30]. TMB-8 ($100 \mu\text{M}$) did not affect the oleate-induced translocation of PtdOH phosphatase activity to membranes in the post-mitochondrial supernatant ($47 \pm 5.5\%$ of activity in membrane fraction after treatment with 0.5 mM -oleate).

Effects of TMB-8 on choline transport and metabolism

Transport of choline into N1E-115 cells in the presence of TMB-8 was investigated by measuring the short-term (20 min) uptake of labelled choline into the intracellular water-soluble precursor pool as a function of choline concentration in the medium. Under these conditions, [^3H]choline uptake into the water-soluble cytosolic compartment was linear for at least 1 h at both high and low choline concentrations, and less than 2% of the intracellular radioactivity was found in phospholipids. Choline and phosphocholine were the only water-soluble metabolites labelled. Choline entered N1E-115 cells both by diffusion and by a relatively low-affinity specific uptake that exhibited saturation kinetics (Fig. 4). The diffusion component was measured by incubating cells at 0°C and also by incubating cells in the presence of an excess of unlabelled choline (50 mM) as described by Diamond & Kennedy [31]. Both methods gave the same result. As reported previously [32], diffusion represented 11% of total choline uptake at the highest concentration of choline in the medium ($150 \mu\text{M}$), but only 3.5% of choline uptake at the usual concentration of choline in DMEM ($28 \mu\text{M}$). The K_t (concentration for half-maximal specific transport of choline) for the saturable component was $20.5 \pm 1.3 \mu\text{M}$ and the V_{max} was $181 \pm 13 \text{ pmol/min per mg}$ of protein (mean $\pm$ S.E.M., $n = 9$). TMB-8 had no effect on uptake of choline by diffusion, but was a competitive inhibitor of choline uptake by the saturable transport system, with an apparent K_i of $10 \mu\text{M}$.

The distribution of radioactivity from [^3H]choline among the intracellular water-soluble metabolites was further investigated after a 20 min pulse as used for kinetic analysis of choline uptake and after a 60 min pulse (Table 4). TMB-8 ($75 \mu\text{M}$) decreased [^3H]choline incorporation into choline and phosphocholine by about 80% at both times, without any significant change in the ratio of radioactive choline to radioactive phosphocholine. TMB-8 did not cause any measurable accumulation of CDP-choline. Similar results were obtained with $75 \mu\text{M}$ -chlorpromazine. Labelling of metabolites was decreased by 40% at 20 min, with no change in the choline/phosphocholine ratio. Labelling was decreased by 60% at 1 h, with a small but significant increase in the choline/phosphocholine ratio, suggesting possible inhibition of choline kinase by chlorpromazine.

TMB-8 and intracellular Ca^{2+}

To determine whether the effects of TMB-8 on choline uptake and phospholipid metabolism in N1E-115 cells were coincident with possible changes in Ca^{2+} homeostasis, the intracellular Ca^{2+} concentration was monitored by using the fluorescent probe fura-2. TMB-8 ($100 \mu\text{M}$) did not alter the resting intracellular Ca^{2+} concentration ($50\text{--}100 \text{ nM}$), but blocked the transient increase in intracellular free Ca^{2+} caused by bradykinin ($10 \mu\text{M}$) in this cell line.

DISCUSSION

Our results show that TMB-8 (at concentrations used to block intracellular Ca^{2+} release) has several effects on the metabolism of phospholipids and their precursors in N1E-115 cells. None of these effects appear to be mediated by Ca^{2+} , since the resting intracellular Ca^{2+} concentration was not altered by TMB-8. TMB-8 acts selectively at the plasma membrane to decrease entry of choline, P_i and inositol, while not affecting ethanolamine or serine uptake. Effects on phosphate and inositol (20 and 35% decrease respectively) were transient and observed only when the labelled precursor and TMB-8 were added at the same time. TMB-8 may block Na^+ channels [33], but this is unlikely to be the mode of action, since transport of serine [34] as well as of inositol [35–37] and phosphate [38] is Na^+ -dependent. Addition of TMB-8 to cell cultures after a prelabelling period did not prevent further uptake of [^{32}P] P_i or [^3H]inositol, and effects of TMB-8 on phospholipid metabolism were separated from initial effects on precursor uptake.

In some respects, alteration of phospholipid metabolism by TMB-8 resembled the effects of propranolol, chlorpromazine and similar cationic amphipathic compounds [39]. Such compounds intercalate into membranes and disrupt membrane conformation at high concentrations. At low concentrations ($10\text{--}200 \mu\text{M}$), they alter phospholipid metabolism by redirecting incorporation of precursors from neutral phospholipids (PtdCho, PtdEtn) formed from diacylglycerol to acidic lipids (CMP-PtdOH, PtdIns, PtdGro) synthesized from PtdOH [24,40]. This has been attributed to inhibition of PtdOH phosphatase, on the basis of observations that chlorpromazine and similar compounds inhibit PtdOH phosphatase activity *in vitro* and in whole cells [24–26]. TMB-8 is a cationic amphipath, but does not inhibit PtdOH phosphatase. Triacylglycerol synthesis as monitored by [^{14}C]oleate incorporation into whole cells was decreased by chlorpromazine, but not by TMB-8. Furthermore, TMB-8 did not inhibit PtdOH phosphatase in an assay *in vitro*, did not alter the distribution between soluble (inactive) and membrane-bound (active) forms of the enzyme and did not counteract oleate-induced translocation of PtdOH phosphatase to membranes. Since N1E-115 cells are dependent on exogenous choline for PtdCho synthesis (PtdEtn is not methylated), and both PtdCho labelling and choline uptake were inhibited to the same extent, the major action of TMB-8 on PtdCho synthesis is probably a decrease in available choline and/or its water-soluble metabolites rather than of diacylglycerol.

Measurement of [^3H]choline incorporation into water-soluble precursors of PtdCho seemed to exclude effects on choline kinase, CTP:phosphocholine cytidylyltransferase and CDP-choline:diacylglycerol cholinephosphotransferase. Although TMB-8 decreased incorporation of choline into intracellular choline and phosphocholine by 80%, the ratio of radioactivity found in these two metabolites was not changed and CDP-choline did not accumulate. Furthermore, the same enzyme phosphorylates choline and ethanolamine in mammalian cells [41,42], yet TMB-8 did not inhibit ethanolamine uptake or PtdEtn synthesis. Most likely the major site of TMB-8 action is choline transport.

Choline transport in mammalian cells occurs by both a high-affinity Na^+ -dependent hemicholinium-3-sensitive system that is coupled to acetylcholine synthesis and a low-affinity system that is relatively Na^+ -independent and insensitive to hemicholinium-3. Both systems are present in cholinergic and in non-cholinergic adrenergic neuroblastoma cell lines [43]. Our K_t value ($20 \mu\text{M}$) for the saturable component of choline transport is similar to values reported for low-affinity choline transport in several neuroblastoma clones, including N1E-115 [32]. Kinetic data indicated

that TMB-8 is a competitive inhibitor of this transport system. However, maximum inhibition achieved was only 80%. High-affinity transport capacity at approx. 10% of total choline transport in neural cells [43,44] and diffusion (4%) account for most of the TMB-8-insensitive choline uptake. Choline and ethanolamine share the same high-affinity Na⁺-dependent hemicholinium-3-sensitive transport system, but have separate low-affinity transport systems in endothelial cells [45]. Since ethanolamine uptake was not decreased, TMB-8 may specifically block low-affinity choline transport and be useful as a selective inhibitor.

An alternative explanation would require choline uptake and PtdCho synthesis to be so tightly coupled that inhibition at any site in the pathway would decrease labelling of all intermediates and PtdCho to a similar extent. In actively proliferating transformed cells, entering choline is immediately converted into PtdCho such that the kinetics of choline transport and PtdCho synthesis are the same [42]. Such an explanation is also consistent with our finding that TMB-8 added to N1E-115 cells preincubated with [³H]choline blocked further entry of labelled choline into water-soluble precursors and into PtdCho to a similar extent, even though the water-soluble fraction contained a substantial quantity of prelabelled precursors. Although interpretation is complicated by possible increased degradation of prelabelled PtdCho, the data suggest that a major portion of pre-existing water-soluble metabolites was not available for PtdCho synthesis. Functional channelling of transported choline to PtdCho in the presence of added intracellular precursors has been reported for electroporated glioma cells [46].

TMB-8 effects on PtdEtn labelling depended on the experimental protocol. [³²P]P_i incorporation into PtdEtn was not affected, except at high TMB-8 concentrations. Since DMEM does not contain ethanolamine, synthesis of PtdEtn *de novo* measured this way depends on serine exchange with existing PtdEtn to provide endogenous ethanolamine. TMB-8 did not affect ethanolamine transport or PtdEtn labelling when added at the same time as labelled ethanolamine, but decreased labelling of PtdEtn in cells prelabelled with [¹⁴C]ethanolamine without affecting the apparent availability of labelled precursors. TMB-8 probably caused loss of prelabelled PtdEtn by stimulating serine exchange, since the concentration-dependence of increased [³H]serine incorporation into PtdSer and of decreased PtdEtn labelling from [¹⁴C]ethanolamine were similar (linear up to at least 150 μM), whereas effects of TMB-8 on other phospholipids were more evident at low concentrations. Thus it is unlikely that TMB-8 has any direct effect on PtdEtn metabolism.

Like many other cationic amphipathic compounds, TMB-8 stimulated PtdIns synthesis. Chlorpromazine and other cationic drugs stimulate inositol exchange in C6 glioma cells [24], but not in rat brain preparations [47]. TMB-8 does not affect inositol exchange in N1E-115 cells, since the same maximum stimulation of PtdIns formation was observed with [³H]inositol, which measures both exchange and synthesis *de novo*, as well as with [³²P]P_i, which only measures synthesis *de novo*. Unlike propranolol in pineal glands [40], inositol competition with glycerol phosphate for CMP-PtdOH does not limit TMB-8-stimulated PtdIns synthesis in N1E-115 cells, since inositol was available in the medium and radioactive inositol in the cytosol was not decreased by TMB-8. TMB-8-stimulated synthesis of CMP-PtdOH and PtdIns in N1E-115 cells was similar to the effects of propranolol in pineal glands. However, the increase in PtdGro labelling was 10-fold greater, suggesting that TMB-8 may selectively increase PtdGro synthesis.

TMB-8 cannot be considered a general antagonist of intracellular Ca²⁺ mobilization, since membrane conductances for cations may be altered [33,48], Ca²⁺ influx rather than in-

tracellular release may be decreased [49–51] or intracellular Ca²⁺ may be mobilized in some cell lines [6,52,53]. Results obtained with TMB-8 must also be interpreted with caution, even in cells exhibiting selective blockage of intracellular Ca²⁺ release. In addition to the effects on choline transport and phospholipid metabolism described here, other Ca²⁺-independent effects of TMB-8 have also been reported for oxidative metabolism [9], ketogenesis [10] and superoxide production [8]. The mechanism of action is not known in these instances, and altered phospholipid metabolism may underlie some of the effects described. Caution is particularly important in studies of phospholipid-mediated signal transduction involving degradation of PtdCho by phospholipase C or phospholipase D. By influencing PtdCho turnover so dramatically, TMB-8 may interfere with cell signalling by both Ca²⁺-dependent and Ca²⁺-independent mechanisms.

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