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Inhibition by vitamin E of drug accumulation and of phospholipidosis induced by desipramine and other cationic amphiphilic drugs in human cultured cells

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1 Cationic amphiphilic drugs (CADs) are widely used in chronic pharmacotherapies in spite of frequently observed side effects connected with lysosomal phospholipid (PL) storage.

2 It has recently been shown that α-tocopherol (α-Toc) inhibits drug- and PL accumulation in cell cultures chronically exposed to the CAD, amiodarone.

3 The mechanisms of α-Toc action on drug kinetics and PL storage were studied in human cultured fibroblasts exposed to single and repetitive doses of desipramine and other CADs.

4 α-Toc did not influence the initial, pH-dependent rapid phase of drug uptake. It inhibited, in a dose-dependent manner, the slow and the cumulative phases of drug uptake and coincidently the accumulation of cellular PLs.

5 The inhibitory effects of α-Toc on CAD and PL accumulations depends on the ratio between CAD and α-Toc concentrations in the medium. This points to competition between α-Toc and CADs for PL complex formation.

6 Effectiveness of α-Toc on drug uptake varies among different CADs. It depends on its structural integrity but is independent of stereoisomerism. The inhibitory action is restricted to the piggyback slow drug uptake and therefore related to the proportion of membrane-mediated transport to permeation into lysosomes (rapid uptake). This proportion differs among CADs.

7 α-Toc prevents lysosomal membrane-PL storage, accelerates disintegration of PL-stores and normalizes drug-related increased membrane fluidity. This strongly suggests that α-Toc restores membrane recycling, impaired by CAD exposure.

8 It remains to be tested *in vivo* whether α-Toc reduces CAD side effects without interfering with drug effectiveness.

Keywords: Vitamin E = D-α-tocopherol; cationic amphiphilic drugs; desipramine; cellular drug accumulation; phospholipids; lysosomes; cell culture

Introduction

Cationic amphiphilic drugs (CADs) represent compounds of different therapeutic classes such as antidepressants, neuroleptics, and antiarrhythmics. In their neutral, lipophilic form CADs enter cells and their organelles. In acidic cellular compartments these drugs become efficiently protonated and thus trapped in, e.g. lysosomes (De Duve *et al.*, 1974; Ohkuma & Poole, 1978; Ferrari & Cutler, 1990). As a result of pH-dependent ion trapping, total lysosomal drug concentrations may exceed extracellular levels by orders of magnitude (de Duve *et al.*, 1974). This explains the remarkable intracellular accumulation of weak amphiphilic bases *in vitro* and *in vivo*. Lysosomotropic drugs may inhibit lysosomal phospholipid (PL) metabolism leading to the formation of dense cytoplasmic granules, i.e. lysosomes filled with undegraded PLs (Lüllmann *et al.*, 1978). CADs either directly inhibit lysosomal phospholipases (Hostetler *et al.*, 1988) or form undegradable complexes with PLs (Lüllmann & Wehling, 1979; Joshi *et al.*, 1989). The formation of drug-PL complexes further enhances intracellular accumulation of drugs (Hein *et al.*, 1990; Reasor, 1991). Side effects that occur after chronic therapy with CADs are mostly accompanied by lysosomal phospholipidosis. However, a causal relationship between side effects and phospholipidosis has yet to be proven (Adams *et al.*, 1986; Kodavanti & Mehendale, 1990).

D-α-Tocopherol (vitamin E, α-Toc) has been shown to inhibit efficiently cellular drug accumulation and concomitant phospholipidosis during exposure of cultured cells to amiodarone (Amio) and desethylamiodarone (DEA) (Honegger *et al.*, 1995). In the present study we investigated the mechanism and specificity of the actions of α-Toc, its derivatives and of other lipophilic antioxidants on cellular kinetics and effects of several CADs *in vitro*. The following CADs were used: desipramine (DMI), chlorpromazine (CPZ), chloroquine (CQ), and propranolol (Prop). Special consideration was given to DMI, since its pharmacokinetics (Honegger *et al.*, 1983), its subcellular localization (Stoffel *et al.*, 1987), and its effects on PL metabolism (Fauster *et al.*, 1983) have been documented previously in fibroblasts.

Methods

Cell cultures

Human skin fibroblasts were obtained from biopsies of healthy persons at minor surgical interventions after informed consent. Cells were cultured in Eagle's minimal essential medium supplemented with 10% foetal calf serum (Honegger *et al.*, 1993). For experiments fibroblast monolayers were grown to confluency in glass Petri dishes (diameter 5 cm; cell protein 0.3 mg; medium 3 ml per dish).

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Drugs and chemicals

Tritiated CPZ, DMI and Prop (specific radioactivities, 20 to 70 Ci $mmol^{-1}$) were purchased from Du Pont de Nemours (NEN), Switzerland. ^{14}C-labelled and non-labelled Amio and DEA (specific radioactivities: 31.2 and 35.8 mCi $mmol^{-1}$, respectively) were generously provided by Sanofi S.A. France. Cell culture media and chemicals of p.a. grade were from Sigma, Switzerland and Merck Chemicals, Germany.

Drug uptake experiments

Cells were exposed to single or multiple doses of CADs (CQ or radiolabelled [^{14}C]-Amio, [^{14}C]-DEA, [^{3}H]-CPZ, [^{3}H]-DMI, [^{3}H]-Prop) with or without α-Toc. Uptake of radiolabelled CADs was measured as previously described (Honegger *et al.*, 1983). The pH-dependent cellular uptake of CADs was inhibited with 20 mM ammonium chloride (Ohkuma & Poole, 1978).

Fluorimetrical determination of chloroquine

Culture medium or acidic ethanolic extracts of cells were adjusted to pH 13–14 with NaOH and extracted twice with diethyl ether. Ether phases were pooled and extracted with 0.1 M HCl. The resulting lower phase was mixed with 1 M NaOH (5:1) and fluorescence intensity was determined (excitation 331 nm, emission 386 nm).

Membrane fluidity

Fluidity of plasma membranes was estimated with fluorescence polarization technique (Hermetter *et al.*, 1989). Trimethylammonium-diphenylhexatriene (TMA-DPH) was used as a fluorescence marker. Fluorescence anisotropy, an inverse measure for membrane fluidity, was determined in monolayer cell cultures on coverslips as recently described by Toplak *et al.* (1990).

Determination of cellular α-tocopherol

Cells were washed twice with culture medium before harvesting. α-Toc was extracted from cell homogenates with ethanol/hexane (1 + 1), and determined spectrophotometrically according to Desai (1984). In order to test for possible oxidation of the vitamin during incubation, samples of incubation media, both from cell-containing and cell-free dishes, were also collected and analysed.

Equilibrium dialysis

Binding of DMI to culture medium (adjusted to pH 7.4 and to a final phosphate buffer concentration of 30 mM) was determined with a Dianorm two-chamber dialysis apparatus (Weder & Bickel, 1970). Chambers were separated by a Visking regenerated cellulose membrane (mol. wt. cut-off 12,000). One ml of buffer with 1–20 μM radiolabelled DMI was introduced into the first, 1 ml medium into the second chamber and allowed to equilibrate during 4 h at 37°C with gentle rotation (20 r.p.m.). Drug concentrations were determined in both chambers. To obtain net binding, the concentration of DMI in buffer (first chamber) was subtracted from the total concentration of DMI in medium (second chamber).

Protein and phospholipids

Proteins were determined according to the method of Lowry *et al.* (1951). Total PLs were determined according to Van Veldhoven & Mannaerts (1987). Briefly, PLs were precipitated with 1.2 M trichloroacetic acid and digested in a mixture of 14% perchloric acid and 2 M sulphuric acid (1 + 9). PL-phosphate was then determined spectrophotometrically with malachite green reagent at 610 nm.

Results

Kinetics of cellular uptake of desipramine in the absence of α-tocopherol

Single dose uptake kinetics Initial drug uptake was rapid (Figure 1a, open symbols). The rate was about 1.0 nmol $min^{-1}\,mg^{-1}$ of cell protein during the first 5 min. After 2 h of exposure to an initial concentration of 5 μM DMI cells accumulated about 10 nmol mg^{-1} protein. This corresponded to an enrichment factor of approximately 300 between intra- and extra-cellular DMI concentrations. A slower linear phase of uptake was observed until the end of the experiment (28 h). The presence of 20 mM ammonium chloride reduced the uptake of 5 μM DMI by about 50% at 2 h and totally blocked further DMI uptake (Figure 1b open triangles).

Chronic uptake of DMI (Figure 2, open circles) Fibroblasts were repetitively exposed to media with 5 μM DMI by changing the media every 48 h. Fibroblasts strongly accumulated the drug, reaching a total intracellular concentration of 64 nmol DMI mg^{-1} of protein after 8 days of exposure. The amount of net DMI uptake slightly decreased with each repetitive dose but no saturation of drug uptake was observed for the observation period of 16 days (Figure 3, open circles).

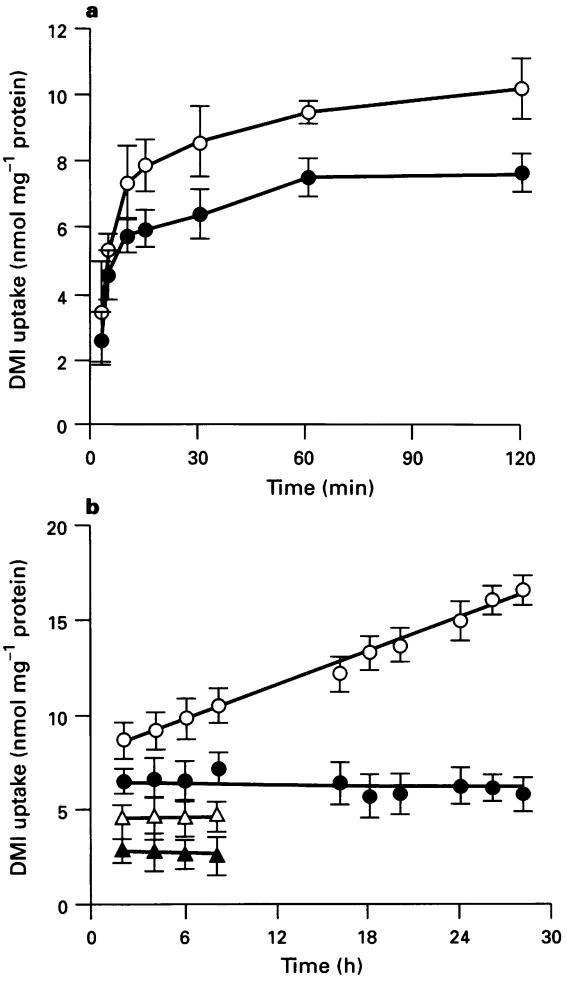

Figure 1 Kinetics of single-dose uptake of DMI. Confluent fibroblast monolayers were exposed to 5 μM [^{3}H]-DMI alone (○), to 5 μM [^{3}H]-DMI in the presence of: 50 μM α-Toc (●); 20 mM ammonium chloride (△), or 20 mM ammonium chloride and 50 μM α-Toc (▲). Drug uptake was measured from 5 min to 2 h (a) or from 2 h to 28 h (b). Symbols represent means ± s.d. of 6 (a) or 12 (b) separate cultures.

Effects of α-tocopherol on cellular desipramine uptake

Single dose uptake kinetics Pretreatment of cells with 100 μM α-Toc for 48 h reduced the subsequent rapid phase of DMI uptake, in the absence of α-Toc, by 25%. The subsequent slow phase of uptake, however, was not affected (results not shown).

Simultaneous addition of 50 μM α-Toc and 5 μM DMI led to an inhibition of the initial DMI uptake by 25% and to a total suppression of the following linear phase of uptake (Figures 1a,b, closed circles) after 60 min. The inhibitory effect of a simultaneous addition of α-Toc on DMI uptake was the same whether it was preceded by an α-Toc pretreatment or not (results not shown).

Inhibitory effects of α-Toc and ammonium chloride on DMI uptake were additive (Figure 1b, closed triangles).

Chronic uptake α-Toc (50 μM) completely inhibited the chronic cumulative uptake of 5 μM DMI (Figure 2a). The extent of inhibition of drug accumulation was related to the ratio between DMI and α-Toc concentrations. Half maximal inhibition of DMI storage was achieved when cells were exposed to 10 μM DMI and 50 μM α-Toc, or to 5 μM DMI and 20 μM α-Toc, or to 2 μM DMI and less than 10 μM α-Toc (Figure 2a, Table 1).

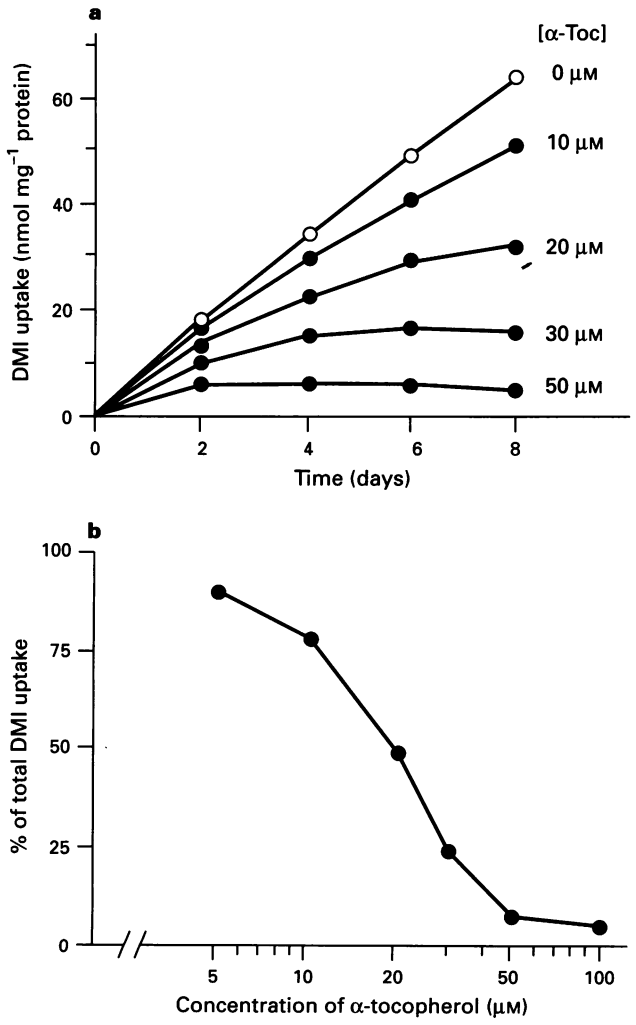

Figure 2 Effects of α-Toc on the kinetics of chronic uptake of DMI. Confluent fibroblast monolayers were exposed during 8 days to 4 repetitive doses of 5 μM [^{3}H]-DMI in the absence (○) or in the presence (●) of 5 to 50 μM α-Toc. Drug uptake was determined at the end of each 48 h interval; s.d. were within the symbols ($n \geq 4$). (a) Uptake kinetics; (b) total drug uptake expressed in % of drug uptake without α-Toc.

Effect of temperature on the effectiveness of α-Toc on DMI uptake Cell cultures were exposed to 5 μM DMI in the presence and absence of 50 μM α-Toc at different temperatures. The inhibitory effect of α-Toc on the slow phase of DMI uptake was strongly temperature-dependent. It was seen only above 20°C and then increased with increasing temperatures (Figure 3).

Effect of α-Toc on previously accumulated DMI In fibroblasts chronically pretreated with 5 μM DMI for 8 days, co-administration of 50 μM α-Toc to the culture medium at day 8 induced a net efflux of the drug (Figure 4, closed circles). After 4 changes of the α-Toc containing medium in 48 h intervals more than 90% of DMI, previously taken up, was released from the cells in spite of the continued presence of DMI in the medium. Cells that were further exposed to DMI alone (Figure 4, open circles) continued to accumulate the drug.

Effects of derivatives of α-tocopherol and other antioxidants on DMI uptake

Derivatives of α-Toc and other lipophilic antioxidants were tested for their interactions with DMI uptake (Table 1). Both, the reducing head group and the lipophilic membrane anchoring side chain of α-Toc were functionally relevant. Slight structural modifications of the chromanol head group such as partial demethylation (β-;γ-Toc) or acetylation (tocopheryl acetate) as well as replacement of the lipophilic phytyl side chain with a hydrophilic moiety (Trolox-C) resulted in a loss of inhibition of DMI uptake. A marked but not total reduction of effectiveness was observed with oxidized α-Toc (tocopherylquinone) which may have been partially reduced during incubation of cultured cells. The racemic DL-form was as effective as the natural D-isomer of α-Toc. Other lipophilic antioxidants (probucol, butylated hydroxytoluene or retinol) had no effect on DMI uptake.

Binding of desipramine to culture medium

Equilibrium dialysis studies revealed that the fraction of 5 μM DMI bound to components of regular culture medium was 25%; 37% was bound in culture medium supplemented with 50 μM α-Toc. α-Toc (50 μM) thus decreased the concentration of free DMI by 16%.

Uptake of α-tocopherol

α-Toc solubilized in culture medium was partially oxidized when kept under incubation conditions in cell-free Petri dishes. The medium concentration of the non-oxidized form decreased by 27% in 48 h. In contrast, no oxidation of α-Toc was detected when it was added to fibroblast cultures. This indicates that oxidative degradation was inhibited in the presence of cell monolayers. Uptake of 50 μM α-Toc was increased in cells with DMI-induced phospholipidosis. The relative increase in α-Toc uptake was identical to the increase in PL content. This suggested a direct effect of cellular PL levels on α-Toc accumulation.

Neutralization of DMI-induced membrane fluidization by α-tocopherol

Membrane anisotropy in cultured fibroblasts was determined with TMA-DPH as a fluorescence marker. Chronic exposure to DMI led to a dose-dependent reduction. The fluidizing effect of 5 μM DMI was completely reversed by the co-administration of 50 μM α-Toc. α-Toc itself had no significant effect on TMA-DPH sensitive membrane fluorescence anisotropy (Figure 5).

Influence of α-Toc on chronic uptake of CADs and on CAD-induced phospholipidosis

Effects of 50 μM α-Toc on drug accumulation and on PL storage were studied in cells chronically exposed to 5 μM

Table 1 Effects of α-tocopherol (α-Toc) derivatives and other antioxidants on chronic desipramine (DMI) uptake

Compounds and concentrations		*% DMI uptake* 2 μM DMI	5 μM DMI	10 μM DMI
D-α-Toc	10 μM	23.0±5.1	78.9±2.1	–
	20 μM	7.6±3.8	48.6±2.1	–
	50 μM	–	12.5±3.1	48.7±7.4
DL-α-Toc	20 μM	–	56.9±2.9	–
	50 μM	–	12.0±2.5	–
DL-α-Toc-acetate	50 μM	–	95.4±2.1	–
Trolox-C	50 μM	–	99.2±2.1	–
DL-α-Toc-quinone	10 μM	85.7±3.8	–	–
	20 μM	67.1±3.6	–	–
Probucol	20 μM	–	93.3±2.6	–
All-*trans*-retinol	10 μM	96.8±4.4	–	–
Butylated hydroxytoluene	20 μM	95.5±4.3	–	–
Ethanol	1%	99.5±1.5	100.2±2.0	101.1±2.1

Monolayers of cultured human fibroblasts were exposed during 8 days to media with initial concentrations of 2, 5 or 10 μM radiolabelled DMI in the presence or absence of the different compounds listed in the table. Media were changed every 48 h. Values (mean±s.d., $n \geq 4$) are expressed as % of DMI uptake in absence of antioxidant compounds (=100%).

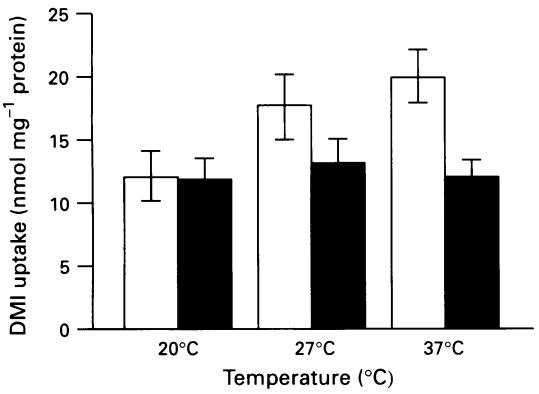

Figure 3 Effect of temperature on the effectiveness of α-Toc on DMI uptake. Confluent fibroblast monolayers were exposed to a single dose of 5 μM [^{3}H]-DMI in the presence (solid columns) and in the absence (open columns) of 50 μM α-Toc at different temperatures (20; 27 and 37°C) for 24 h. Columns represent means s.d.± of 6 separate cultures.

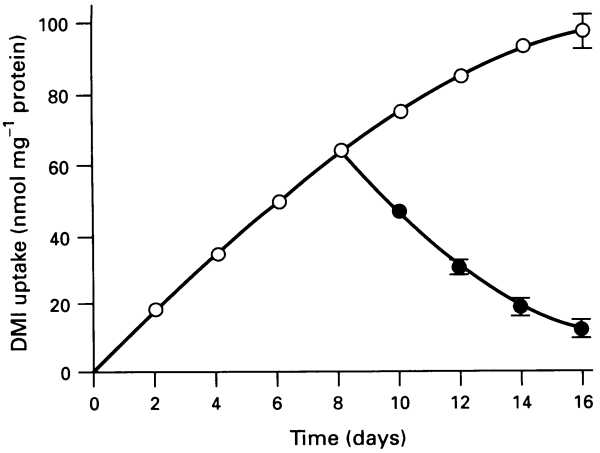

Figure 4 Effect of α-Toc on previously accumulated DMI. Confluent fibroblast monolayers were exposed either eight times for 48 h to 5 μM [^{3}H]-DMI (○) or to four doses of 5 μM [^{3}H]-DMI during the first 8 days, followed by 4 doses of 5 μM [^{3}H]-DMI together with 50 μM α-Toc (●) during the next 8 days. Cumulative DMI uptake was measured at the end of each 48 h interval: s.d. were within the symbols ($n \geq 4$).

concentrations of different CADs (Figure 6). In the case of Amio, 10 μM concentrations were added in order to reach comparable intracellular drug levels to those with 5 μM DEA. Media were changed every 48 h for up to 8 days.

All CADs accumulated in fibroblasts, and uptake never reached saturation. Different extents of drug accumulation were observed, the highest were obtained with DEA (80% of the added 5 μM dose) and with Amio (41% of a 10 μM dose). Intermediate accumulations were seen with CPZ, CQ and DMI (43%, 48% and 41% respectively). The lowest cumulative uptake was obtained with Prop (24%). α-Toc (50 μM) inhibited cellular accumulations of DEA and of DMI by more than 90%, those of Amio, CQ and Prop by 55%, 33%, and 32% respectively. For CPZ, reduction was only 16% (Figure 6a).

Intracellular accumulation of each CAD was accompanied by intracellular storage of PLs (Figure 6b). Amio and DEA caused the highest phospholipidosis, more than doubling normal cellular PL contents (+120%). CPZ and DMI increased PLs by +60% and +57% respectively, whereas phospholipidosis by CQ was low and comparable to that induced by Prop (+34% and +37% respectively). α-Toc reduced the extent of phospholipidosis correspondingly to the decrease in drug accumulation. Reduction exceeded 70% with DEA and DMI, 51%, 43% and 35% with Amio, Prop and CQ respectively and 11% with CPZ.

Morphological changes in phase contrast microscopy

Chronic CAD exposure consistently induced dense cytoplasmic granules. Co-administration of 50 μM α-Toc greatly diminished the formation of these dense bodies (DEA, DMI, Prop>Amio, CQ), except for CPZ (not illustrated).

Discussion

Cellular phospholipidosis is a common observation after chronic administration of CADs *in vivo* as well as *in vitro* (Lüllmann-Rauch, 1979; Kodavanti & Mehendale, 1990). Membrane PLs are stored in lysosomes (Stoffel *et al.*, 1987) since CADs interfere with membrane recycling (Gonzales-Noriega *et al.*, 1980) and with PL degradation in the lysosomal compartment (Fauster *et al.*, 1983). It has been shown that impairment of membrane recycling by CADs is reversible within 3 h. Later it becomes irreversible, and membrane constituents must be synthesized *de novo* (Gonzales-Noriega *et al.*, 1980) and from this moment total PL content will increase. Our data demonstrate that the extent of PL storage correlated with the extent of drug storage. This applied to all of the drugs tested, and indicates that CADs form complexes with PLs, in

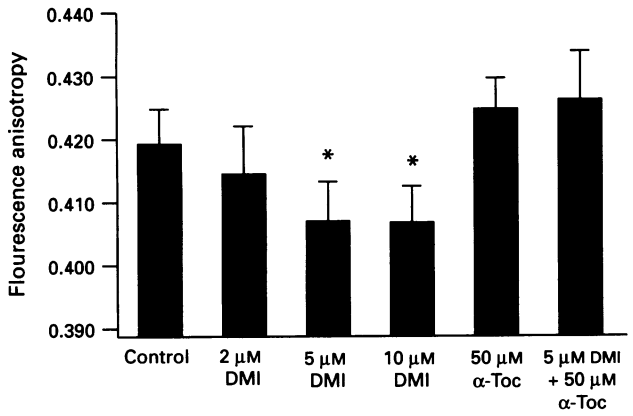

Figure 5 Effects of chronic DMI and of α-Toc on TMA-DPH fluorescence anisotropy in plasma membranes. Confluent fibroblast monolayers grown on cover slips were exposed to 2, 5 and 10 μM DMI respectively; 50 μM α-Toc or 5 μM DMI with 50 μM α-Toc for 8 days with 4 changes of the medium. Fluorescence anisotropy was measured with TMA-DPH as a marker. Columns represent means ± s.d. Significant deviations from control values are marked with an *($P<0.05$).

particular within lysosomes. Complexation lowers intralysosomal free drug concentration and entails further drug uptake (Lüllmann-Rauch, 1979; Joshi *et al.*, 1989).

For all CADs tested, α-Toc inhibited both, drug accumulation and the development of phospholipidosis. The extent of α-Toc interference varied largely between structurally different drugs (DEA, DMI > Amio, CQ, Prop > CPZ), but even between structurally similar amines such as Amio and DEA. Reduced drug uptake was always accompanied by a proportional decrease in drug-induced phospholipidosis. Again, these findings suggest a relationship between the storage of CADs and PLs and an interaction of α-Toc with drug-PL complex formation or with complex stability. The reduction in the free extracellular DMI concentration by the presence of α-Toc in the medium demonstrates that in fact α-Toc binds DMI but that this cannot explain the strong inhibition of the accumulating drug uptake. α-Toc predominantly inhibited the slow and the cumulative phase of DMI uptake. However, it hardly interfered with the initial phase (up to 5 min) of DMI uptake into lysosomes which is driven by the pH-gradient and sensitive to ammonium chloride.

α-Toc is highly lipophilic and distributes in PL bilayers (Fukuzawa *et al.*, 1992). Its uptake into cells was proportional to the PL content.

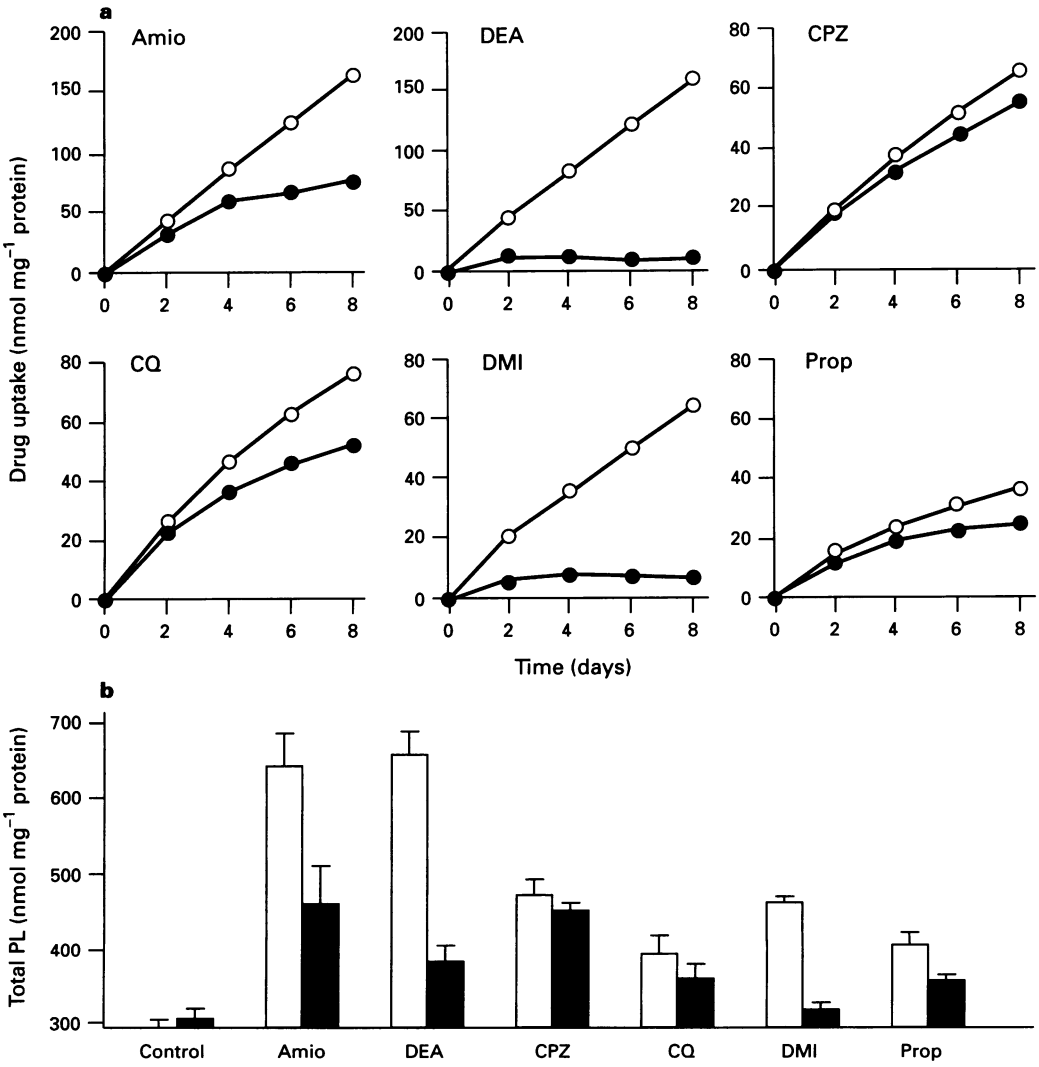

Figure 6 Effects of α-Toc on accumulation of different CADs and on drug-induced phospholipidosis. Confluent fibroblast monolayers were exposed for 8 days to either 10 μM Amio, or to 5 μM DEA, CPZ, CQ, DMI and Prop respectively in the absence (open symbols and columns) or in the presence (solid symbols and columns) of 50 μM α-Toc. Media were changed every 48 h. (a) Cellular drug accumulation was determined every 48 h. Values represent the cumulative drug uptake related to cell protein (s.d. within the symbols, $n \geq 4$). Note the different scales of the ordinate for the uptake of Amio and DEA in comparison with those for the other drugs. (b) Total cell PL were determined at the end of the treatment and values were related to cell protein (mean ± s.d., $n \geq 4$).

Based on our experimental findings we formulate the following hypothesis on the mechanism of action of vitamin E: α-Toc impairs cumulative CAD uptake mediated by piggyback transport and drug-PL complex formation and thereby normalized DMI-induced modifications of membrane properties and abnormal membrane recycling. This hypothesis is supported by the following considerations. DMI is accumulated by free penetration and subsequent trapping in lysosomes (rapid uptake) as well as by complexation with PLs in cellular membranes and subsequent piggyback transport (slow uptake). The presence of DMI results in membrane fluidization which interferes with plasma membrane recycling, resulting in an increased net flux of membranes into the lysosomes. Judged from binding experiments with liposomes, DMI accumulation is further increased in the endosomal/lysosomal compartment since the binding capacity of membrane PL for the drug seems to be enhanced at low pH compared to the binding capacity at the more neutral pH. In addition, in the acidic environment, PL membranes are within a high drug concentration favouring further PL interaction and drug complexation. This situation may also be responsible for the reduced PL turnover in the lysosomes (Lüllmann-Rauch, 1979; Fauster *et al.*, 1983).

α-Toc prevents DMI-PL complex formation and displaces DMI from preformed complexes most likely beginning already in the prelysosomal compartment where DMI has a lower affinity towards PLs. Free DMI released from the membranes is leaving the cells along its concentration-gradient as cellular concentrations are several hundred times higher than the extracellullar concentrations. Membrane internalization and piggyback transport of drugs are energy-dependent mechanisms and thus temperature-controlled. DMI uptake by piggyback transport is decreased by lowering the incubation temperature. At the same time the effectiveness of α-Toc is reduced. This again suggests that α-Toc exclusively interferes with the slow uptake piggyback drug transport as an early event by competing with DMI for the membrane binding. Dissociation of the DMI-membrane PL complexes by α-Toc reverses fluidization of membranes to normal and thus restores membrane recycling even before PL storage has completely returned to normality.

The effectiveness of α-Toc can be explained by its physical properties in cellular membranes and by its competition with CADs leading to normalization of CAD-induced changes in membrane fluidity in the vacuolar compartment and restoring the equilibrium between membrane fusion and membrane fission.

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