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# VITAMIN E REDUCES ACCUMULATION OF AMIODARONE AND DESETHYLAMIODARONE AND INHIBITS PHOSPHOLIPIDOSIS IN CULTURED HUMAN CELLS

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**Abstract**—Chronic administration of amiodarone (AMIO), widely used by clinicians for the treatment of therapy-resistant cardiac arrhythmias, is frequently associated with serious side-effects. AMIO and its main metabolite desethylamiodarone (DEA) are known to induce phospholipidosis *in vivo* and in cultured cells presumably by inhibition of lysosomal phospholipid degradation. D-α-Tocopherol = vitamin E (α-TOC) was able to reduce AMIO and DEA toxicity in cell cultures. Results from the present study showed that α-TOC reduced phospholipidosis in cultured human skin fibroblasts chronically exposed to micromolar concentrations of AMIO and DEA and inhibited cumulative uptake of the drugs in a dose-dependent manner. A linear correlation was observed between cellular AMIO levels and phospholipid accumulation suggesting a stoichiometric relationship. α-TOC was also effective in clearing previously accumulated phospholipids after discontinuation of the drug treatment. The results can best be explained by an interference of α-TOC (a) with drug–phospholipid complex formation responsible for both phospholipid storage and drug accumulation, and (b) with pre-existing drug–phospholipid complexes, accelerating their dissociation and rendering phospholipids to substrates for lysosomal phospholipases. The finding raises hope that side-effects of AMIO and DEA can be prevented or made reversible by the administration of α-TOC. It must, however, be proven that the antiarrhythmic drug will still be effective.

*Key words:* amiodarone; desethylamiodarone; phospholipidosis; α-tocopherol; fibroblast cultures; lysosomes

AMIO§ is a most effective antiarrhythmic drug widely used in the treatment of therapy-resistant ventricular and supraventricular arrhythmia [1–3].

Chronic amiodarone therapy, however, is associated with serious and even fatal side-effects, e.g. pulmonary fibrosis or hepatotoxicity [4, 5]. Most patients develop corneal deposits [6]. Membrane-bound lamellar and granular bodies are found within the cytoplasm of alveolar macrophages, keratocytes and many other cells [7, 8] suggesting a drug-related phospholipidosis [9]. We have recently shown that chronic exposure of cultured human cells to AMIO or its main metabolite DEA induced a dose- and time-dependent PL accumulation with alterations in the cellular and membraneous PL-composition and plasma-membrane function [10].

AMIO and DEA appear to be potent inhibitors of lysosomal phospholipase activities *in vivo* and *in vitro* [11–14]. In addition, both compounds form complexes with cellular PL and the extent of drug accumulation is directly correlated with the amount of cellular PL stores [7, 15]. A causal relationship between drug-induced phospholipidosis and drug toxicity is, however, difficult to prove [16]. AMIO- or DEA-related toxic effects have also been attributed to their potential to form free radicals and to induce lipid peroxidation [17]. Studies by Kachel *et al.* [18] and Ruch *et al.* [19] have shown that the toxic effects of AMIO and DEA on cultured human pulmonary artery endothelial cells and on rat hepatocyte cultures respectively could not be prevented by radical scavenging or antioxidant compounds such as *N*-acetylcysteine and butylated hydroxytoluene, except for α-TOC. This compound was able to time- and dose-dependently reduce AMIO- and DEA-related cell toxicity by an unrelated antioxidant mechanism.

In the present work we investigated the effects of α-TOC on the cellular uptake of AMIO and DEA, on phospholipidosis and on the reversibility of the drug effects in cultured human skin fibroblasts chronically exposed to micromolar concentrations of these compounds. Conditions were comparable to those used in a previous study with AMIO and DEA in cell cultures [10].

## MATERIALS AND METHODS

*Cell cultures.* Skin biopsies for fibroblast cultures were obtained after informed consent from healthy individuals at the occasion of minor surgery. Fibroblast monolayers were grown from the biopsies and cultured in MEM supplemented with 10% foetal

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§ Abbreviations: AMIO, amiodarone (2-butyl-3-(3′-5′-diiodo-4′α-diethylaminoethoxybenzoyl)benzofuran); DEA, desethylamiodarone; α-TOC, D-α-tocopherol = vitamin E; PL, phospholipid; MEM, Eagle's minimal essential medium.

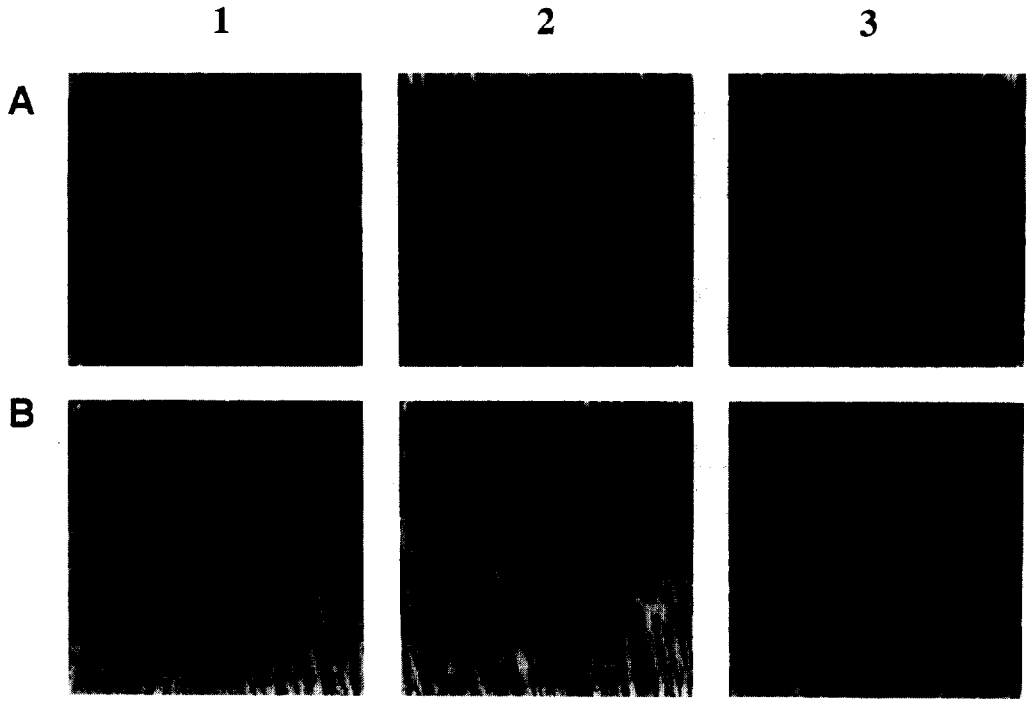


Fig. 1. Morphology of chronically drug-exposed cultured human skin fibroblasts. Confluent cell cultures were exposed to media (MEM) supplemented with 10% foetal calf serum containing 5 μM AMIO or DEA with and without 50 μM α-TOC for 8 days with four medium changes. Control cells were equally exposed to drug-free culture medium with and without α-TOC. Pictures were made by inverted phase microscopy. Calibration bar—50 μM. A1: control; B1: 50 μM α-TOC; A2: 5 μM AMIO; B2: 5 μM AMIO + 50 μM α-TOC; A3: 5 μM DEA; B3: 5 μM DEA + 50 μM α-TOC.

calf serum and buffered at pH 7.4 with bicarbonate and 5% $CO_2$ in air. Fibroblast stock cultures were maintained at 37° in 175 $cm^2$ Falcon culture flasks and the medium was changed twice a week. For experimental purposes, cells were subcultured by trypsinization, seeded into glass Petri dishes at a density of $5 \times 10^3$ cells/$cm^2$ and grown to confluency.

*Uptake studies.* Confluent monolayers of human skin fibroblasts grown in glass Petri dishes (diameter 5 cm) were exposed for 8 days to four doses of [$^{14}$C]-AMIO or [$^{14}$C]-DEA in the absence or presence of increasing concentrations of α-TOC. Stock solutions of all compounds were prepared with ethanol. Final ethanol concentrations never exceeded 0.5% and appropriate amounts were added to control media. Drug doses and experimental conditions are described in detail in the Results section and in the legends to the figures. Generally, cell cultures were exposed to 3 mL of drug containing MEM supplemented with 10% foetal calf serum. Cellular drug uptake was calculated from the decrease in radioactivity in the culture media during the experiments. In addition radioactivity was measured at the end of the uptake period in the acidic cell extracts (1 M HCl:ethanol = 1:10). Possible artefacts due to evaporation of the media or to absorption of the drug to the glass were excluded by incubating cell-free culture dishes with drug containing media under identical conditions. Results were expressed in nmol of drug per mg of cellular protein.

*Protein and PL.* Cellular protein content was determined by the procedure of Lowry *et al.* [20].

Total PL were measured according to Van Veldhoven and Mannaerts [21]. PL were denaturated with trichloroacetic acid and PL phosphorus was oxidized in a mixture of perchloric acid (14%) and 2 M sulphuric acid (1 + 9). PL-phosphate was spectrophotometrically determined with malachite green reagents at 610 nm.

*Reversibility of phospholipidosis.* Confluent fibroblast cultures were exposed to media containing 5 μM AMIO and DEA respectively for 8 days with four changes of the media. Control cells were equally treated with culture medium alone. At the end of this exposure period the media were removed and the cultures rinsed with Hanks balanced salt solution. The 'recovery-phase' was then started by adding either culture medium or medium containing 50 μM α-TOC to the drug pretreated or control cultures. The media were changed daily. Cellular protein and PL contents were determined at the end of the drug exposure and at days 2, 4 and 6 of the recovery period.

*Materials.* Non-labelled and [$^{14}$C]-AMIO and [$^{14}$C]-DEA (spec. radioactivity: 31.2 and 35.8 mCi/mmol, respectively) were kindly provided by Sanofi SA (Paris, France). α-TOC was purchased from

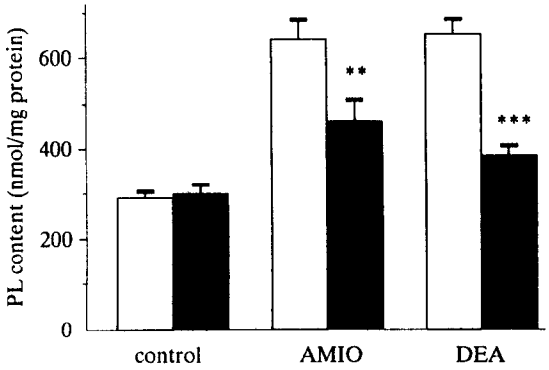


Fig. 2. Phospholipid contents of chronically drug-exposed cultured human skin fibroblasts. Cell cultures were chronically exposed as described in the legend to Fig. 1. Total PL contents were expressed as nmol PL per mg cellular protein in the absence (open columns) and in the presence (closed columns) of 50 $\mu$M $\alpha$-TOC. Values represent mean ± SD of six individual culture plates (**P < 0.01; ***P < 0.001).

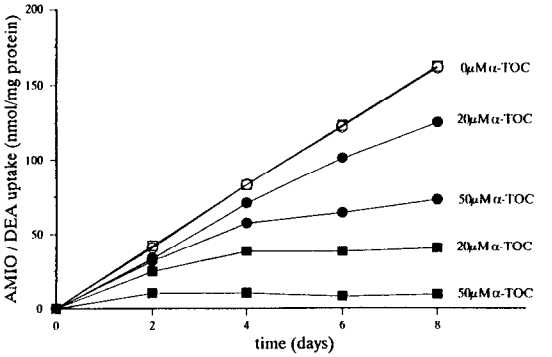


Fig. 3. Reduction of cumulative uptake of AMIO and DEA, respectively, by increasing concentrations of $\alpha$-TOC. Confluent skin fibroblast cultures were exposed for 8 days with four changes of media containing 10 $\mu$M AMIO (○; ●) or 5 $\mu$M DEA (□; ■) in the absence and the presence of 20 or 50 $\mu$M $\alpha$-TOC. Drug uptake was expressed as nmol per mg cellular protein. Final drug accumulations were 162 ± 4 and 163 ± 3 nmol/mg protein for 10 $\mu$M AMIO and 5 $\mu$M DEA, respectively.

Sigma (St. Louis, MO, U.S.A.). The compound was kept under $N_2$, light protected at −22° and periodically tested for oxidative impurities. MEM as powdered culture medium with Earle's salt was obtained from Seromed (Munich, Germany). Tissue culture flasks were from Falcon (Becton and Dickinson, Basel, Switzerland). All other chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany) or from Sigma.

## RESULTS

Chronic exposure of confluent fibroblast cultures to 5 $\mu$M AMIO and DEA, respectively, produced a granular morphology of the cells with numerous perinuclear cytoplasmic dense bodies. These morphological changes were diminished in cells exposed to the drugs in the presence of 50 $\mu$M $\alpha$-TOC (Fig. 1). Chronic exposure to 5 $\mu$M AMIO resulted in a significant increase in cellular PL content. The presence of 50 $\mu$M $\alpha$-TOC in the drug containing media significantly attenuated the drug-induced PL accumulation particularly in DEA-treated cultures (Fig. 2).

The extent of PL accumulation and the amount of drug uptake were similar in cells either exposed to 10 $\mu$M AMIO or 5 $\mu$M DEA. Increasing concentrations of $\alpha$-TOC diminished the cellular uptake of AMIO and DEA. Kinetic experiments showed that the inhibitory potency of $\alpha$-TOC on drug uptake increased with the number of repetitive drug doses (Fig. 3). $\alpha$-TOC was more potent in reducing the accumulation of DEA than of AMIO (Fig. 4).

$\alpha$-TOC caused a dose-dependent proportionate decrease of both stored PL and accumulated drug. The observed changes were linear in the range of 40–140 nmol intracellular AMIO and 100–300 nmol PL per mg of cell protein respectively (Fig. 5). The PL content of cells chronically exposed to 5 $\mu$M AMIO or DEA was twice that of non-treated cells.

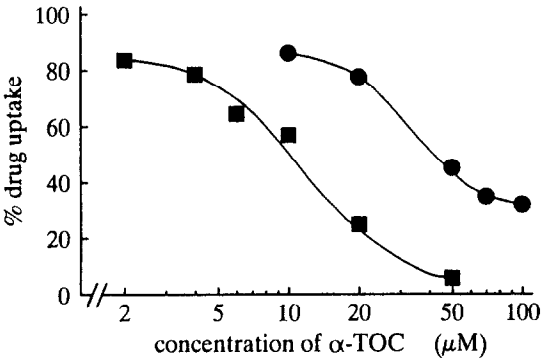


Fig. 4. Potency of $\alpha$-TOC in reducing cumulative uptake of AMIO (●) and DEA(■), respectively. Confluent skin fibroblast cultures were exposed for 8 days with four changes of media containing 10 $\mu$M AMIO or 5 $\mu$M DEA in the presence of increasing concentrations of $\alpha$-TOC. Drug uptake was expressed in percent of drug accumulation in absence of $\alpha$-TOC (= 100%).

Elevated amounts of PL remained unchanged up to 6 days after discontinuation of drug exposure in spite of daily changes of the drug-free medium (Fig. 6). If identically drug pre-exposed cultures were incubated with culture media containing 50 $\mu$M $\alpha$-TOC the elevated PL content declined during the recovery phase and approached values of non-exposed control cells.

## DISCUSSION

As summarized in a review by Vrobel *et al.* [22] the occurrence of severe and even fatal side effects greatly limits the clinical use of AMIO. The toxic side-effects seem to be related to cellular accumulation of the parent drug and its main

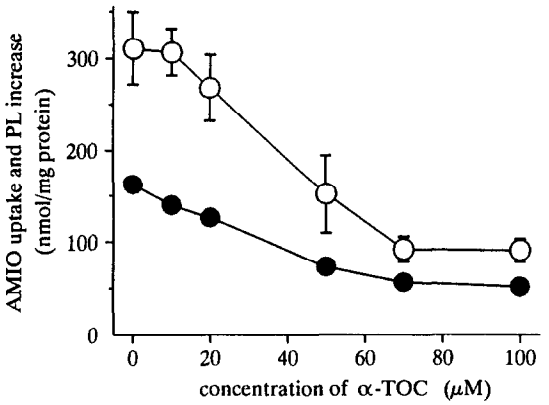


Fig. 5. Cellular AMIO content and PL accumulation. Drug concentrations and PL contents were measured in cultured cells chronically exposed (8 days, four media changes) to 10 μM AMIO in combination with increasing concentrations of α-TOC. Values of absolute cellular AMIO (●) correspond to those in Fig. 4. Data on net PL accumulation (○); i.e. difference between PL contents of drug-exposed and untreated control cells, were expressed in nmol PL per mg of protein. Values represent mean ± SD of six cultures per condition. SD of AMIO concentrations were again within the size of the symbols.

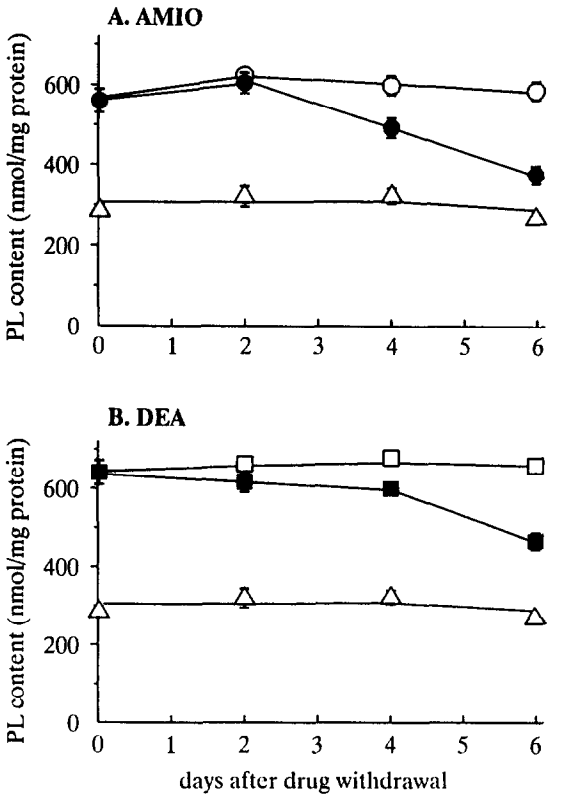


Fig. 6. Reversibility of AMIO- or DEA-induced PL accumulation in the absence and the presence of α-TOC. Cultures were exposed for 8 days with four changes of media containing 5 μM AMIO (A) or 5 μM DEA (B) in the absence of α-TOC. The drug-containing media were removed and the cells further incubated in media with (closed symbols) and without (open symbols) 50 μM α-TOC. The media were changed daily. Cellular PL were determined up to 6 days after discontinuation of drug exposure. PL content was also measured in untreated control cells (△). Values represent mean ± SD of six parallel cultures. SD are drawn when bigger than the size of the symbols.

metabolite DEA together with the storage of PL in the lysosomal compartment [23]. Cumulative uptake of AMIO and DEA as well as PL storage have also been observed in cultured fibroblasts chronically exposed to low drug doses [10]. High dose toxicity in cultured cells has been reduced by micromolar concentrations of α-TOC exclusively, by mechanisms which presumably are unrelated to its reducing or radical scavenging properties. Other lipophilic antioxidants such as butylated hydroxytoluene or probucol failed to reduce drug toxicity [18, 19].

In our experiments chronic coadministration of α-TOC to 5 μM AMIO or DEA diminished or suppressed granular cytoplasmic inclusions in cultured human fibroblasts and reduced PL accumulation which was the biochemical equivalent to the morphological alterations. Changes in cellular PL composition following chronic exposures to AMIO or DEA [10] were abolished by simultaneous α-TOC administration (results not shown). The suppression of drug-induced PL changes by α-TOC were presumably due to the dose-dependent reduction in cumulative uptake of AMIO and DEA. The reduction was more pronounced for DEA, which may be important in the light of the higher toxicity of this metabolite [10, 19]. Cell exposure to 10 μM DEA led to cell death within 3 days while cells exposed to a combination of 10 μM DEA and 50 μM α-TOC survived longer than 3 weeks. This indicates that the presence of α-TOC not only reduces drug accumulation and phospholipidosis but also consecutive toxic effects of AMIO and DEA. The decrease in PL accumulation was dose-dependent and strictly related to the reduction in the intracellular AMIO content suggesting a firm link between the two parameters. In chronically drug-exposed cells lysosomal PL storage persisted unchanged over a drug-free recovery period of more than 6 days while addition of 50 μM α-TOC during the recovery period led to a clearance of the stored PL.

α-TOC was rapidly accumulated within the cells up to 15 times the concentration in the medium. Cellular content of α-TOC was determined from the reducing capacity of cell-extracted α-TOC (unpublished results). The drugs as well as the lipophilic α-TOC concentrate in the same subcellular compartments with high PL storage [24]. Our results as well as the previously observed reduction in acute AMIO and DEA toxicity by α-TOC may be explained by the prevention of intracellular cumulative drug uptake. AMIO and DEA appear to form stable complexes with PL [25]. This accounts for the linear correlation between PL and drug accumulation observed. α-TOC may interfere with this stable complex formation by modifying the binding

equilibrium between drug and PL in favour of the free drug, which is then released.

AMIO has been found to inhibit lysosomal phospholipases $A_1$ and $A_2$ [14] resulting in a strong interference with PL degradation in cultured cells as well as *in vivo*. The observed PL storage may thus originate from direct inhibition of the enzymes by the free drug or from drug–PL complexes that become undegradable. An interference of $\alpha$-TOC with this stable complex formation would reduce intralysosomal drug cumulation while reactivating PL degradation at the same time. It would also explain the increased clearance of stored PL in chronically AMIO-pretreated cells. Furthermore, $\alpha$-TOC may restore impaired membrane recycling by normalizing membrane fluidity. Our observations raise expectations that $\alpha$-TOC might not only prevent the occurrence of side-effects but might also accelerate the disappearance of existing side-effects in chronically AMIO-treated patients. In chronically AMIO- and DEA-exposed fibroblasts the accumulated PL showed modified patterns in whole cells and in isolated plasma membrane preparations [10]. The changes were accompanied by increased membrane fluidity and reduced $\beta$-adrenoceptor transmission, which are considered to be part of the desired rather than of the unwanted drug effects. It remains to be clinically proven whether a reduction in drug and PL accumulation and in accompanying membrane effects by $\alpha$-TOC coadministration will also modify or even abolish clinical drug effectiveness *in vivo*.

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