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CELLULAR ACCUMULATION OF AMIODARONE AND DESETHYLAMIODARONE IN CULTURED HUMAN CELLS

CONSEQUENCES OF DRUG ACCUMULATION ON CELLULAR LIPID METABOLISM AND PLASMA MEMBRANE PROPERTIES OF CHRONICALLY EXPOSED CELLS

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Abstract—Amiodarone (AMIO), a potent antiarrhythmic drug, is clinically widely used despite its frequent side effects after chronic administration. These side effects coincide with an intralysosomal accumulation of AMIO and its main metabolite desethylamiodarone (DEA) and may be causally related to the drug-induced intracellular storage of phospholipids (PL). Kinetics of cellular uptake and release of radiolabelled AMIO and DEA were studied following single and multiple exposures of cultured human skin fibroblasts to 5 and 10 μM drug concentrations. AMIO and DEA were efficiently taken up into cultured cells. The rate of uptake was slower than that of other cationic amphiphilic drugs. The intracellular steady state concentrations were in the millimolar range suggesting a lysosomal trapping. Repetitive exposures of cultures resulted in a cumulative and partly saturable drug uptake. The accumulation of DEA was higher than that of AMIO throughout. AMIO and DEA previously taken up into the cells during a 2 hr exposure were completely released into the washing media, suggesting an exchangeable form of the accumulated drugs. Following repetitive exposures only part of the drugs was released. Under chasing conditions using washing media containing non-labelled AMIO and DEA respectively or ammonium chloride the release of the chronically accumulated ^{14}C-labelled drugs was increased. This suggested a drug storage in the form of complexes in acidic compartments. Phospholipid (PL) content as well as individual PL fractions were changed in whole cells and in isolated plasma membranes. PL accumulation is assumed to occur by inhibition of PL degradation due to formation of non-degradable drug–PL complexes or by inhibition of phospholipase activities. Cellular PL accumulation seemed to interfere with PL recycling. Changes in PL composition of purified plasma membranes were in part complementary to the ones in whole cells. The alterations in membrane PL composition may explain the changes in membrane fluidity and the decrease in β-adrenoceptor density and in isoproterenol-stimulated cAMP formation. The results obtained provide an explanation for the pharmacokinetic, and possibly for the pharmacodynamic and also toxicological behaviour of AMIO and DEA *in vivo*.

Amiodarone (AMIO‡) originally used in angina pectoris therapy has since become a most effective antiarrhythmic drug [1], which is used to treat therapy-resistant arrhythmias of ventricular and supraventricular origin [2]. AMIO and its main metabolite desethylamiodarone (DEA) are both strongly bound to plasma proteins [3]. Part of AMIO is also stored in membrane bilayers of circulating erythrocytes [4, 5]. Tissue concentrations of AMIO and of DEA are 100 to above 1000 times higher than the corresponding plasma concentrations [6]. Organs that store these drugs are adipose tissue, liver and lung, but also skin, pancreas, myocard and thyroid gland. Except for fat, the tissue concentrations of the metabolite are higher than that of the parent drug following chronic administration of AMIO [7, 8]. The mean elimination half-life is more than 40 days and varies considerably between individuals [6, 9].

AMIO therapy suffers from a number of sometimes severe side effects [2]. The organs that may suffer from adverse effects are lung, liver, nervous system, skin, thyroid gland and cornea, but rarely heart [10]. Skin is affected most frequently [11]. After long-term administration of AMIO reaching cumulative doses of over 40 g the skin of patients shows an increased sensitivity to sunlight. The manifestation may range from increased tanning to severe burns after exposure to sunlight containing UV-A. Following prolonged therapy with daily doses over 600 mg and/or after a minimal cumulative dose of about 160 g a grey–blue pigmentation of the skin

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‡ Abbreviations: AMIO, amiodarone; DEA, desethylamiodarone; TMA-DPH, 1-[4-(trimethyl-amino-phenyl]-6-phenylhexa-1,3,5-triene; IL-1, interleukin 1; MEM, minimal essential medium; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PMV, plasma membrane vesicles; PL, phospholipids; HPTLC, high pressure thin layer chromatography; CGP 12177, [4-(3-tert. butylamino-2-hydroxypropoxy)-benzimidazole-2-on hydrochloride]; IBMX, 3-isobutyl-1-methylxanthin; SPH, sphingomyelin; PA, phosphatidic acid; PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamin; PS, phosphatidylserin; CAD, cationic amphiphilic drug.

may persist over weeks [11, 12]. Common to all clinical side effects reported are massive storage of AMIO and DEA together with cellular phospholipidosis. In light and electron microscopy multilamellar lysosomal inclusions can be observed [8, 13]. AMIO belongs to the group of cationic amphiphilic substances such as chloroquine, imipramine, chlorpromazine and others which accumulate in lysosomes of cells and tissues [14]. From many of these substances phospholipid accumulation has been reported as one of the complications of prolonged drug administration. AMIO has been shown *in vivo* and in cultured cells to interfere with phospholipid metabolism [15–17]. DEA is even more effective in this respect [18–21].

Since many functional aspects regarding the side effects of AMIO remain unanswered kinetics of cellular uptake and release of AMIO and DEA were studied following single and multiple exposures of cultured human skin fibroblasts to 5 and 10 μM drug concentrations. Changes of phospholipid content as well as of individual phospholipid fractions were measured in whole cells and in isolated plasma membranes following chronic drug exposure. Changes of membrane functions were assessed in terms of β-adrenoceptor densities and by the isoproterenol-stimulated cyclic AMP response. Finally, cellular membrane fluidity was measured by membrane fluorescence anisotropy using 1-[4-(trimethyl-amino)-phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH) as a marker.

MATERIALS AND METHODS

Tissue cultures. Skin biopsies for fibroblast cultures were obtained after informed consent from healthy persons at the occasion of minor surgery. Fibroblast monolayers were grown from the biopsies and cultured as described previously [22].

Macrophages were cultured from a spontaneously transformed monocyte/macrophage cell line that was obtained from peripheral blood monocytes in our laboratory. The cells, which are tetraploid, grow attached to the glass culture material and are partially contact inhibited. They secrete interleukin 1 (IL-1), phagocytose latex particles and possess a high density of β-adrenoceptors [23].

Uptake and release studies. Confluent monolayers of human skin fibroblasts grown in glass petri dishes (diameter 5 cm) were exposed to single or multiple doses of radiolabelled amiodarone ([^{14}C]AMIO) and desethylamiodarone ([^{14}C]DEA), respectively. Drug concentrations and experimental conditions are indicated in the Results and in the legends to the figures. Experiments were started by exposing the cell cultures to 3 mL of drug containing Eagle's minimal essential medium (MEM) supplemented with 10% foetal calf serum. Cellular drug uptake was calculated from the decrease of radioactivity in the media during the experiments. At the end of the uptake period the amount of radioactivity taken up into the cells was measured following acidic extraction of the drug (1 N HCl : EtOH = 1:10). Possible artefacts due to evaporation of the media or to adsorption 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 nanomoles per milligram of cellular protein.

Drug release from monolayer cultures was determined at the end of the uptake period. After removal of the labelled medium the cultures were quickly rinsed with cold Hank's solution. Drug-free MEM (3 mL) was added to the cultures and the cells were incubated for 1 hr at 37°. This procedure was repeated three times in 1 hr intervals. The amount of drug released from the cells was determined by measuring the radioactivity in an aliquot of the "washing media". In chase experiments the drug-loaded cell cultures were incubated four times for 1 hr with MEM containing 20 mM ammonium chloride and AMIO or DEA, respectively. Details have been described by Honegger *et al.* [24].

Isolation of plasma membranes. The isolation of fibroblast plasma membranes was achieved by a modification of the method of Scott [25]. In essence, cells were grown to confluence in 175-cm^2 Falcon culture flasks. The monolayer cultures were washed with phosphate-buffered saline (PBS) and subsequently incubated at 37° with 25 mM formaldehyde and 2 mM dithiothreitol in Ca^{2+} and Mg^{2+} containing PBS for 90 min [26]. The plasma membrane vesicles (PMV) formed were washed away from the cells three times with 10 mL of isotonic (140 mM NACl) Tris–HCl buffer (10 mM), pH 7.4, containing 0.1% of bovine serum albumin (BSA). Detached cells and cellular debris were separated from the vesicles by centrifugation (500 *g*). The PMV were collected by centrifugation at 48,000 *g*. The pellets were washed and recentrifuged in hypotonic Tris buffer to lyse the PMV and to remove soluble proteins. The final pellet was stored at −20° prior to analyses.

Protein and phospholipids (PL). Proteins were measured with the BCA protein assay reagent (Pierce Chemical Co., Rockford, IL, U.S.A.).

DNA measurements were performed according to the method of Hinegardner [27] using bovine thymus DNA as a standard.

Total cholesterol was enzymatically determined with a test kit from Boehringer GmbH (Mannheim, F.R.G.).

Total PL were determined according to Van Veldhoven and Mannaerts [28]. PL were denatured with trichloroacetic acid and PL-phosphorous was oxidized in a mixture of perchloric acid (14%) and 2 N sulphuric acid (1 + 9). PL-phosphate was spectrophotometrically determined with malachite-green reagent at 610 nm. For the isolation and quantification of the individual PL, total cellular lipids were extracted from whole cells or PMV homogenates according to Folch *et al.* [29]. Aliquots of the lipid extracts were applied to silica gel–HPTLC plates (No. 5641, Merck, Darmstadt, F.R.G.). The plates were developed with a solvent mixture of chloroform, methylacetate, propanol-2, methanol and KCl 0.74% in water (25:25:25:10:9, by vol.) for 30 min [30], dried (5 min at 180°) and subsequently cooled for 5 min. Spots corresponding to individual PL were visualized by immersing the plates into Cu(II)acetate (0.5 g/100 mL ethanol) for 10 sec and into H_3PO_4 (20.7 mL of 85% H_3PO_4 in

220 mL methanol), for another 10 sec. After heating (6 min at 180°) the PL-spots were quantified by reflection densitometry (Camag TLC scanner I, Muttenz, Switzerland) according to Kolarovic and Traitler (personal communication).

Fluorescence anisotropy (TMA-DPH). Fluorescence anisotropy as an indirect measure for membrane fluidity was determined in monolayer cell cultures on glass coverslips as recently described by Toplak *et al.* [31].

β-Adrenoceptor-binding assay. Direct radioligand receptor binding studies on intact cells have been performed as previously described by Honegger *et al.* [23]. Macrophages suspended in Hank's solution were incubated with increasing concentrations (0.5–6.0 nM) of the radiolabelled [^{3}H]CGP 12177 [4-(3-tert. butylamino-2-hydroxypropoxy)-benzimidazole-2-on hydrochloride], a specific, hydrophilic β-adrenoceptor blocking agent (Amersham, U.K.). Non-specific binding was determined in the presence of 1 μM timolol, a potent β-adrenergic antagonist. Incubations at 37° were stopped after 60 min by dilution with cold Hank's solution and immediate filtration under controlled vacuum pressure through presoaked glass fibre filters (Whatman GF/C). Cells were digested overnight with Solutron (Kontron AG, Switzerland) and radioactivity was determined in a Kontron Betamatic I Liquid scintillation counter.

cAMP. Macrophage monolayer cultures were preincubated for 15 min with 1 μM isobutyl-1-methylxanthine (IBMX). Stimulation was achieved with 10 μM isoproterenol for exactly 10 min at 37°. Cellular cAMP was extracted, lyophilized and determined by means of a specific protein binding assay using a test kit from Amersham.

Chemicals. Nonlabelled and ^{14}C-radiolabelled AMIO and DEA (sp. radioact.: 31.2 and 35.8 mCi/mmol, respectively) were kindly provided by Sanofi SA (Paris, France). MEM dry powdered media with Earle's salt were purchased from Seromed (Munich, F.R.G.). TMA-DPH was obtained from Molecular Probes, Inc. (Eugene, OR, U.S.A.) and kept as 2 mM ethanolic stock solution in the dark at 4°. Glass coverslips were obtained from Assistent (Altnau, Switzerland). All other chemicals, solutions and solvents were of analytical purity grade and were purchased from Merck.

RESULTS

Uptake experiments

Media containing 5 or 10 μM radiolabelled AMIO and DEA, respectively, were added to confluent fibroblast cultures. In short-term experiments cell monolayers were exposed up to 120 min. Drug uptake was non-linear as the uptake rate decreased with time. The amount of intracellular AMIO after 120 min of incubation correlated with the concentrations in the media. At 5 and 10 μM AMIO the intracellular concentrations were 2.75 and 5.5 mM which represented a 550-fold increase above the initial extracellular concentrations. During the 2 hr incubation period extracellular drug concentrations declined to 3.77 ± 0.3 (5 μM) and 7.42 ± 0.2 μM (10 μM), respectively. Cellular enrichment of DEA was higher than that of AMIO. The intracellular concentration of DEA reached 800 times the level of the added drug solution. The extracellular DEA concentrations were reduced to 3.28 ± 0.1 (5 μM) and 5.89 ± 0.3 μM (10 μM) at the end of the 2 hr uptake period. Thus, the indicated ratios between intracellular and added extracellular drug concentrations were in fact 30 (AMIO) to 40% (DEA) higher at steady state condition (Fig. 1a and b).

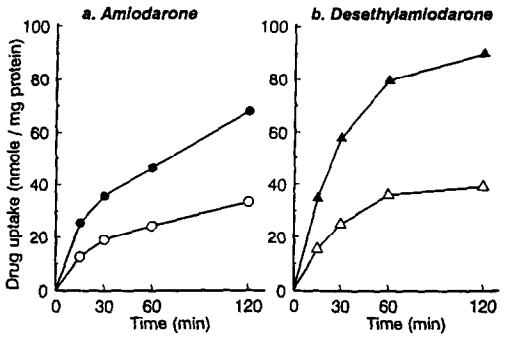

Fig. 1. Uptake of a single dose of (a) AMIO or (b) DEA into confluent cultured fibroblasts. Uptake of AMIO and DEA into confluent fibroblast monolayers was determined following short-term exposures of the cells to culture media containing initially 5 (open symbols) or 10 μM (closed symbols) radiolabelled AMIO and DEA, respectively. Intracellular drug contents were calculated by measuring the decrease in radioactivity in the medium and by determining radioactivity in the cell extracts at the end of the experiments. Data are expressed in nmol of intracellular drug per mg of cell protein.

Cellular uptake after three repetitive 24 hr

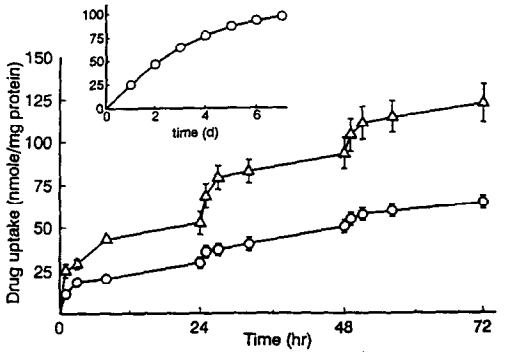

Fig. 2. Uptake of three repetitive doses of 5 μM AMIO or DEA into confluent cultured fibroblasts. Confluent monolayer cultures were exposed three times for 24 hr to 5 μM AMIO (○) or DEA (△). Radiolabelled drug-containing media were renewed every 24 hr and net uptake into cells was measured at time points indicated by determining radioactivity in the media. The curves represent uptake kinetics in nmol of intracellular drug per mg of cellular protein and time of exposure. Inset: cumulative uptake of seven daily doses of 5 μM AMIO.

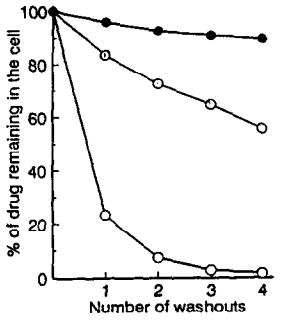

Fig. 3. Release of cellular AMIO after uptake of single or repetitive drug doses. Confluent monolayer cultures were exposed to media containing 5 μM radiolabelled AMIO (initial concentration) for 2 hr (single dose, ○) or 3 (◎) and 7 (●) days with daily changes of the media (repetitive doses). At the end of the uptake periods the media were removed, the cultures quickly rinsed with cold Hank's solution and then fresh drug-free medium was added four times for 1 hr. Cultures were constantly kept at 37° in a 5% CO_2 incubator. Release of radiolabelled drug was measured in the media and is expressed in per cent of the cellular drug amount determined at the end of the uptake period.

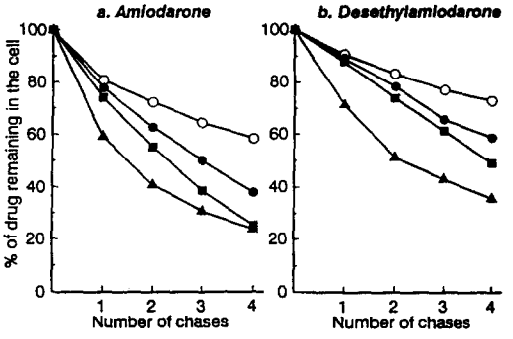

Fig. 4. Chased release of cellular (a) AMIO and (b) DEA after uptake of three repetitive daily doses. Confluent monolayer cultures were exposed to 5 μM radiolabelled AMIO and DEA, respectively, for 3 days as described in the legend to Fig. 3. The chased release of radiolabelled drug was measured in drug-free medium (○) and in media containing 10 μM concentrations of non-labelled AMIO (●) and DEA (■), respectively, or 20 mM ammonium chloride (▲). Media were changed four times in 1 hr intervals. Release of the previously accumulated drugs is expressed in per cent of the cellular amount of drug determined at the end of the uptake period.

incubations was cumulative for AMIO and even more for DEA as shown in Fig. 2. The individual uptake kinetics again were non-linear and did not reach an equilibrium within 24 hr.

The cumulative uptake of AMIO after three individual doses was about half that of DEA. After each additional incubation the fractional uptake decreased indicating a tendency to saturation of cellular content. Following seven daily doses the cumulative intracellular accumulation reached more than five times the amount of the initial 24 hr uptake. Cumulative uptake at 10 μM extracellular AMIO was twice that at 5 μM extracellular concentrations (results not shown).

Experiments with 10 μM DEA under identical conditions showed an even higher cellular accumulation, however, the cells were severely damaged, thus we did not further pursue experiments with chronic DEA exposures of higher than 5 μM concentrations.

Release experiments

After single exposure of confluent cultures to 5 μM AMIO (initial concentration) for 2 hr all of the drug taken up could be removed with four changes of drug-free washout medium. After repetitive daily incubations during periods of 3 or 7 days the drugs accumulated were much more slowly released. Following 3 and 7 days of drug exposure 55% and 90% of AMIO, respectively, were retained intracellularly after four changes of drug-free medium (Fig. 3). The presence of 10 μM non-labelled AMIO or DEA in the washout media (= chase media) increased the cellular release of the accumulated radiolabelled drugs. The chasing potency of DEA was higher than that of AMIO. Addition of 20 mM of the lysosomotropic agent ammonium chloride to the media was most effective in chasing accumulated drugs (Fig. 4a and b).

Lipids

Chronic exposure of confluent monolayer cultures to four repetitive doses of AMIO during a period of 7 days resulted in dose-dependent increases in the cellular contents of PL if related to DNA and protein. The increase in the ratio of PL to protein was larger than the one of PL to DNA, thus the protein to DNA ratio was smaller than in control cells. The changes were more pronounced in DEA-exposed cultures than in AMIO-treated ones (Table 1). In chronically drug-exposed cells (5 μM) not only the PL to protein ratio increased but also the ratio

Table 1. Effects of chronic drug exposures on the ratios between cellular contents of protein, PL and DNA

Ratio	Amiodarone 5 μM	Amiodarone 10 μM	DEA 5 μM
PL/protein	179.3*	194.0*	185.7*
PL/DNA	138.9†	155.8	139.2†
Protein/DNA	68.2*	71.8*	70.6*

Confluent monolayer cultures were exposed for 7 days to four repetitive doses of AMIO at concentrations of 5 and 10 μM and of DEA at 5 μM, respectively. Before and after exposures the contents of DNA, protein and PL per culture were measured. The ratios between these three cellular parameters are expressed in per cent of those of control cultures.

Data represent results of three individual experiments with three plates per condition (*$P < 0.01$; †$P < 0.05$).

Table 2. Effects of chronic drug exposures on the ratios between the contents of protein, PL and cholesterol in whole cells and purified plasma membrane vesicles

	Amiodarone		DEA	
Ratio	Whole cells	PMV	Whole cells	PMV
PL/protein	180.4*	71.5*	188.3*	61.6*
CHOL/protein	140.0*	78.0*	144.3	64.3†
CHOL/PL	77.8*	109.5†	77.8	104.1

Confluent monolayer cultures were exposed to four repetitive doses of 5 μM AMIO and DEA, respectively, for 7 days. Protein, PL and cholesterol (CHOL) contents were determined in whole cells and in highly purified PMV. PL/protein, CHOL/protein and CHOL/PL ratios are expressed in per cent of those of control cultures.

Data represent values of five and three individual experiments with AMIO and DEA, respectively (*$P < 0.01$; †$P < 0.05$).

between cholesterol and protein was enhanced. The quotient of cholesterol to PL was decreased. In highly purified PMV of chronically drug-exposed cells the ratios between protein, cholesterol and PL were changed but in the opposite direction to those in the whole cells (Table 2).

The PL compositions in chronically drug-exposed cells were altered greatly compared to those of non-exposed control cells. The qualitative deviations in the individual PL of exposed cells from that of control cells were the same for AMIO and DEA. In whole cells the relative changes consisted of marked decreases in sphingomyelin (SPH) and phosphatidic acid (PA) while phosphatidylcholine (PC) and more so phosphatidylinositol (PI) were increased. The extents of the observed drug-induced changes in the cellular PL composition were dose dependent. Concentrations of 0.5 μM DEA induced significant alterations (results not shown). In purified PMV of the drug-exposed cells the percentage deviations of the individual PL were quantitatively and in part also qualitatively different from whole cells (Fig. 5).

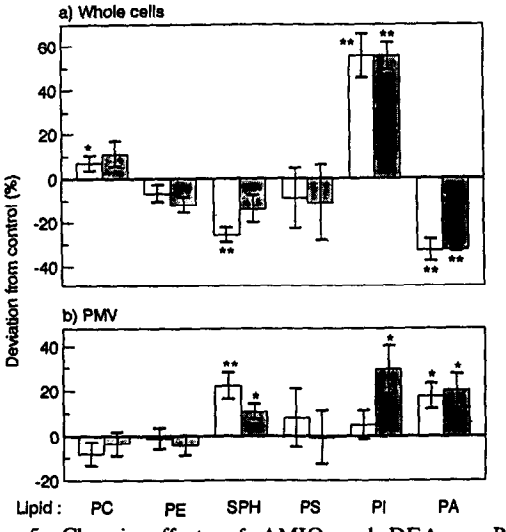

Fig. 5. Chronic effects of AMIO and DEA on PL compositions of whole cells and PMV of exposed cells. Confluent monolayer cultures were exposed to four repetitive doses of 5 μM AMIO (open columns) or DEA (shaded columns) for 7 days. PL were extracted from isolated PMV and from whole cells. Individual PL were separated and quantified on HPTLC plates. Percentage deviation of individual PL of drug-exposed cells from untreated controls are shown. Data represent results from three individual experiments (**$P < 0.01$, *$P < 0.05$).

Membrane fluidity

Membrane fluidities of control and of drug-exposed cells were estimated as fluorescence anisotropy using TMA-DPH as a marker. Cell monolayers on coverslips were treated identically to cultures in petri dishes. Repetitive but not single exposures of cells to 5 μM concentrations of AMIO or DEA resulted in decreases in anisotropy which became significant between three and seven doses for both drugs. This suggested an increase in fluidity of the plasma membranes of chronically drug-exposed cells (Fig. 6).

β-Adrenergic transmission

β-Adrenergic transmission representing a membrane function was measured by determining β-adrenoceptor density and isoproterenol-stimulated cAMP formation. Cultured human macrophages were exposed to drugs as described above for fibroblasts. Chronic exposures (four feedings, 7 days) of this cell-type to 5 μM AMIO resulted in a significant reduction in the receptor density of 24.9 ± 1.5% or in absolute B_{max} values from 20.69 ± 0.9 fmol/mg cell protein in control cells to 15.52 ± 0.4 fmol/mg in drug-exposed macrophages. The equilibrium dissociation constant (K_D) of 0.97 ± 0.3 nM in control cells was not changed by the AMIO exposition. The drug-induced reduction of the isoproterenol-stimulated cAMP response was more pronounced than that of the receptor density. The β-adrenoceptor-dependent cAMP formation was decreased by 48.4 ± 9.9% from that of control cells.

DISCUSSION

AMIO and its main metabolite DEA were

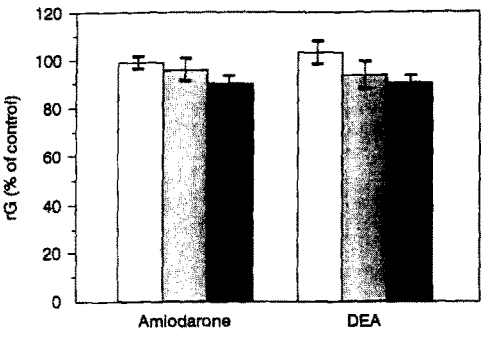

Fig. 6. Effects of AMIO and DEA on TMA-DPH fluorescence anisotropy of drug-exposed cells. Fibroblasts were cultured on coverslips until confluency and were then exposed to one (open columns); three (shaded columns); and seven (closed columns) daily doses of 5 μM AMIO or DEA. Fluorescence anisotropy (rG) was measured directly under steady state conditions by placing the coverslips in cuvettes containing 5 μM TMA-DPH in Hank's solution. Fluorescence anisotropy as a measure for membrane fluidity is expressed in per cent of control values (100%) obtained from cells exposed to drug-free media.

efficiently taken up into the cultured cells. The rate of uptake was slower than those of other cationic amphiphilic drugs (CAD) such as desipramine or chloroquine. The amount of drug taken up after 15 min of exposure was 30–40% for AMIO and DEA of the respective values after 2 hr of exposure. In comparable studies, more than 90% of desipramine and chloroquine were taken up at 15 min [24, 32]. The extent of intracellular steady state concentrations following exposure to micromolar concentrations of AMIO and DEA was, however, in the same millimolar range as for other CADs. Cellular drug uptake reduced extracellular drug concentrations by 30–40%. Thus, ratios between intracellular concentrations and drug concentrations in the starting media should be corrected by the factor of starting conc/steady state conc of the media. In spite of the slower rate of uptake, the extent of intracellular accumulation suggested a lysosomal trapping similar to that observed with other lysosomotropic drugs [33]. Direct and indirect evidence for intralysosomal localization have been presented for AMIO and DEA *in vivo* and *in vitro* [34, 35]. Repetitive exposures of cell cultures to AMIO and DEA respectively resulted in a cumulative and partially saturable drug uptake. Under all conditions tested DEA accumulation was always higher than that of AMIO. This may explain the cellular toxicity observed with DEA concentrations above 5 μM. The higher degree of DEA uptake may be a consequence of increased affinity of DEA to the storage site. The slower uptake rates of AMIO and DEA compared to those of other CADs could either be explained by a reduced membrane permeability for the drugs or by an alternative drug uptake mechanism, e.g. binding of the drugs to the cell surface followed by membrane internalization. This could explain both the slower rate of uptake and possibly the cytotoxicity.

AMIO and DEA previously taken up into cells during a single 2 hr exposure was completely released into washing media. This suggested that the drugs were still in an exchangeable form and were released according to the concentration gradient. After multiple drug exposures only part of the drugs was released from the cells. Thus, the drug retention was no longer subject to the concentration gradient alone. This may be due to complex formation of the drugs with PL and/or drug storage in cellular compartments less accessible to the concentration gradient. Under chasing conditions using media containing non-labelled AMIO and DEA respectively or ammonium chloride, the release of the chronically accumulated labelled drugs was substantially increased. This indicated that part of the stored drugs was still in an exchangeable form and was located in an acidic compartment. This supported the hypothesis that AMIO and DEA form complexes with PL which are stored in lysosomal compartments. The higher chasing potency of DEA as well as its increased cellular retention are suggestive for a higher complex affinity of the metabolite than that of the parent drug AMIO. This is in agreement with the preferential tissue accumulation and the longer elimination half-life of DEA following chronic treatment with AMIO *in vivo* [6].

Addition of ammonium chloride to the culture medium raises the pH value in cellular acidic compartments. After dissociation, the non-protonated form NH_3 which permeates cellular membranes becomes protonated and trapped in the low pH environment of endosomal/lysosomal vesicles as NH_4^+ and thus raises the vesicular pH [36]. It may exert its effect on drug release by pH-dependent dissociation of the drug–PL complexes. But even after chasing the cells with four media changes 30–40% of the chronically accumulated drugs still remained intracellularly. This indicates a further storage site which is inaccessible to drug gradients, to chasing and to quenching of the acid pH. This storage site could apply to tertiary non-acidic lysosomes. Lysosomal storage of AMIO and DEA as well as phospholipidosis has been observed in guinea pigs after chronic treatments with AMIO [37]. Morphological evidence has been in favour of lysosomal storage of drug–PL complexes.

PL accumulation in AMIO- and DEA-exposed fibroblasts is assumed to occur by inhibition of PL-degradation as has been demonstrated already with desipramine and other CADs in cultured cells [38]. Activities of lysosomal phospholipases may be directly inhibited by the drugs as suggested by Hostetler *et al.* [17] or indirectly by raising the intralysosomal pH value above the acidic pH optima of the enzymes or by the formation of drug–PL complexes that are no longer substrates for the phospholipases [13]. The fact that cholesterol, the other main membrane lipid constituent was not increased in AMIO- and DEA-exposed cells to the same extent as PL favoured the hypothesis that PL were not stored as part of intact membranes. This was also supported by the typical disproportionate

changes in the PL patterns of drug-exposed whole cells.

Cellular PL accumulation seemed to interfere with PL turnover and recycling since the changes in PL composition of purified plasma membranes were somewhat complementary to the ones in whole cells.

The composition of the membrane lipid matrix is one important factor for the regulation of membrane fluidity. Therefore, the changes in the membrane PL found in chronically AMIO- and DEA-exposed skin fibroblasts could explain the observed increase in membrane fluidity measured as a decrease in fluorescence anisotropy. Repetitive but not single exposures of cells to 5 μM AMIO or DEA reduced TMA-DPH fluorescence anisotropy. Thus, the presence of the drugs in the membrane *per se* did not change membrane fluidity. The alteration observed in the plasma membrane seemed to be the result of an adaptive modification in the lipid composition following repetitive and prolonged drug exposures. Links between plasma membrane lipid compositions and fluorescence anisotropy have been found for drug [31] and for temperature [39] induced PL changes.

Functional integrity of plasma membranes is dependent on the protein and lipid environment. Cultured fibroblasts have a low density of β-adrenoceptors and thus alterations in receptor number are difficult to assess in these cells. We, therefore, exposed cultured human macrophages to AMIO and DEA, respectively. A significant reduction in β-adrenoceptor density as well as in isoproterenol-stimulated cAMP formation was found in chronically drug-exposed macrophages. Since these changes in β-adrenergic transmission were accompanied by an alteration in the PL pattern comparable to that seen in fibroblasts, a causal relationship between the composition of the PL matrix and the receptor–effector coupling may be discussed.

In summary, the cellular drug accumulation and the accompanying drug-induced changes in the PL metabolism of cultured cells resulted in cellular PL storage and a change in the plasma membrane PL composition. This provides explanations for the pharmacokinetic and possibly for the pharmacodynamic behaviour as well as for the pathophysiology of the observed adverse effects of AMIO and even more of DEA in skin and in other organs *in vivo*.

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