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Identification of cyclosporin binding sites in rat liver plasma membranes, isolated hepatocytes, and hepatoma cells by photoaffinity labeling using [^{3}H]cyclosporin-diaziridine

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[^{3}H]Cyclosporin diaziridine, a new photoaffinity label, enters rat liver cells in the dark. Photoaffinity labeling of isolated rat liver-cell plasma membranes with this probe modifies several polypeptides with molecular mass of 200, 85, 54, 50, 34 kDa. The major labeled protein of 85 kDa represents 2% of the total plasmamembrane protein. A 50 kDa protein is heavily labeled in freshly isolated rat hepatocytes at low temperature and after short incubation in the dark. The 85 kDa protein becomes substituted after longer preincubation periods at temperatures above 10°C. This suggests a localisation at the cytoplasmic side of the membrane. Several controls point to a specific interaction with the above mentioned proteins. Comparison of [^{3}H]cyclosporin-diaziridine- and isothiocyanatobenzamido[^{3}H] cholic acid-labeled membrane proteins reveals identity of binding proteins with the exception of the 85 kDa protein. However, the interaction of bile acids with the 85 kDa protein became apparent at higher concentrations as demonstrated by the differential photoaffinity labeling experiments. In the cytosol of rat liver cells, further [^{3}H]cyclosporin-diaziridine binding proteins could be identified. In particular, a 17 kDa polypeptide was found which appears similar to cyclophilin, a protein known to be present in T-lymphocytes (R. Handschumacher et al. (1984) Science 226, 544–547: Cyclophilin. A specific cytosolic binding protein for cyclosporin A). Proteins with molecular mass of 90, 56, 30, 24, 20 kDa are labeled in AS-30D ascites hepatoma cells and those with molecular mass of 200, 150, 80, 70, 42, 25 kDa in Ehrlich ascites tumor cells.

Introduction

Cyclosporin A, a fungal metabolite, is being used increasingly for immunosuppression in patients undergoing organ transplantation [1,2]. Certain side effects of this treatment have been well established. A dose-dependent and apparently reversible nephrotoxicity is associated with rising levels of serum creatinin [3]. Hepatotoxicity with elevated serum bilirubin levels was reported in patients with serum cyclosporin concentrations above 400 ng/ml [4]. Cyclosporin is markedly lipid soluble, accumulates within the liver, and undergoes biliary recirculation [5]. Schade et al. [4] reported elevated bile-salt levels in patients receiving cyclosporin A after renal transplantation. No hepatospecific enzyme changes (transaminases or canalicular enzyme levels) could be detected. The nature of the hepatic injury leading to increased bile salt and bilirubin levels can be explained by reduced clearance of bile salts and bilirubin from portal blood. The canalicular part of liver cells seems not to be primarily involved in the mechanism of cholestasis. The effect is probably caused by a non-competitive inhibition of bile acid uptake by cyclosporin A [6]. For identification of

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cyclosporin binding sites on various cell types a photolabile diaziridine derivative of cyclosporin A was synthesized. This photoaffinity label also inhibits the uptake of bile acids by hepatocytes and was used in the present study. Foregoing reports revealed that cyclosporin A interacts with the bile-acid transport system without being transported itself.

In previous studies from our laboratory [7–9] and from other laboratories [10,11], several bile-acid binding and (or) transporting proteins were identified using affinity or photoaffinity probes. Polypeptides with molecular weights of 67, 60, 54, 50 and 37 kDa are possibly involved in binding and transporting bile acids into hepatocytes [7–9]. Cyclosporin binding proteins will be compared with bile-acid binding proteins to evaluate whether cyclosporin might be translocated by one of the bile-salt carriers as demonstrated for phalloidin [12]. The latter system is not present in cell membranes of AS-30D ascites hepatoma cells [9,13] and Ehrlich ascites cells. We therefore tested binding of cyclosporin to AS-30D ascites and Ehrlich cells to determine relevant differences between bile-salt transporting and nontransporting cell types. The results presented suggest that cyclosporin A binds to proteins involved in bile-acid transport. Uptake kinetics however do not support the idea that cyclosporin might be taken up (as is phalloidin) by one of the bile-salt carriers.

Materials

Cyclosporin-diaziridine and [^{3}H]cyclosporin diaziridine (spec. act. 623 mCi/mmol) were generous gifts from Sandoz AG, Basel, Switzerland *. Isothiocyanatobenzamido[^{3}H]cholate was synthesized by Professor Dr. H. Fasold as described previously [9]. The purity of [^{3}H]cyclosporin diaziridine (> 98%) was determined by combined ^{1}H/^{3}H-NMR spectroscopy, reverse-phase HPLC and TLC/radio TLC by Dr. R. Wenger, Sandoz AG, Basel. The purity of isothiocyanatobenzamido[^{3}H]cholate (> 90%) was determined by thin-layer chromatography, melting point and infrared spectrometry. SDS was purchased from Merck, Darmstadt, F.R.G., phenylmethylsulfonyl fluoride, bovine serum albumin, acrylamide, *N*,*N'*-methylenebis(acrylamide) and benzamidine from Serva, Heidelberg, F.R.G. All other reagents were of the highest purity grade commercially available.

* We thank Dr. J. Borel and Dr. R. Wenger, Sandoz AG, Basel, for the provision of [^{3}H]cyclosporin diaziridine, cyclosporin A and cyclosporin diaziridine.

Methods

Preparation of hepatocytes

Liver cells were isolated according to Berry and Friend [14] by perfusion of rat liver with 0.05% collagenase, in a Ca^{2+}-free Krebs-Henseleit buffer. Isolated hepatocytes were equilibrated for 30 min in Tyrode buffer (pH 7.4 at 37°C) in O_2/CO_2 (95 : 5, v/v) atmosphere. Cells were used within 2 h after cell preparation. 80% of the cells were viable as indicated by Trypan blue exclusion.

Plasma membrane preparation

Plasma membranes from rat liver were prepared according to the method of Touster et al. [15] with some modifications [8]. The following protease inhibitors were included in the buffers used: phenylmethylsulfonyl fluoride (500 μM); benzamidine hydrochloride (1000 μM); EDTA (5 mM), leupeptin (5 μg/ml). Membranes from the so called microsome pellet were isolated and stored at −70°C in phosphate-buffered saline (pH 7.4) until use.

Photoaffinity labeling procedure

Labeling of cell or plasma membrane suspensions in Tyrode buffer (pH 7.4) or phosphate-buffered saline (pH 7.4), respectively, was carried out in quartz tubes at room temperature. Photolysis of [^{3}H]cyclosporin-diaziridine was induced by a single high-energy ultraviolet light flash with a duration of 1 ms (the design of the apparatus has been published in a separate paper [21]. The distance of the quartz tube from the ultraviolet light flash was 2 cm. Usually $0.6 \cdot 10^6$ hepatocytes/ml of Tyrode buffer or 0.3 mg of plasma membrane proteins/ml of phoshpate-buffered saline were processed. In the case of hepatoma cells $10 \cdot 10^6$ cells per ml of buffer were used (Tyrode buffer, pH 7.4). Preincubation (variation of time or temperature) occurred in the dark. During this period

the cell suspensions were gassed with carbogen (95% O_2/5% CO_2). After photoaffinity labeling, cells and membranes were extensively washed to remove unbound label. The buffers (pH 7.4, 4°C) contained the protease inhibitors mentioned above plus iodoacetamide (1 mM); *p*-chloromercuribenzoate (10 μM). Protein content was determined according to Lowry et al. [16] using bovine serum albumin as a standard. In some experiments hepatocytes were lysed by sonciation for 5 s on ice. The cytosol was separated from the membrane fraction by 100000 × *g* centrifugation. Affinity labeling of plasma membranes of hepatocytes using isothiocyanatobenzamido[^{3}H] cholate was performed as described earlier [9].

SDS-polyacrylamide gel electrophoresis

Samples of cells or plasma membranes (5–200 μg protein) were boiled for 2 min in the presence of 2% sodium dodecylsulfate, 5% mercaptoethanol and 1% dithiothreitol. Electrophoretic separation was performed on 1.5 mm thick 10% polyacrylamide slab gels and 10% or 15% rod gels (gel system according to Weber and Osborn acrylamide/bisacrylamide (74 : 1, v/v) [17]. Polypeptides were stained by a modified silver staining method [18] or with Coomassie blue. Radiolabeled proteins were visualized by fluorography [19]. Quantitation of the radioactivity bound to the polypeptides was done by slicing gel rods. The 2 mm gel discs were solubilized in Lipoluma/ Lumasolve/ water, heated to 40°C for 4 h and cooled to 4°C. Radioactivity was counted in a Packard Tri-Carb 2660 scintillation counter.

The polypeptides of Coomassie blue stained gel rods were scanned using a Gilford Instruments Spectrophotometer, equipped with a linear transport system.

Results

Properties of [^{3}H]cyclosporin diaziridine

Diaziridines are reagents which upon photolysis form carbenes. The long-wave ultraviolet spectrum of cyclosporin diaziridine shows a maximum at 350 nm, which disappears after irradiation [20]. Under our experimental conditions the maximum at 350 nm was reduced by 70% after a single ultraviolet light flash within 1 ms [21]. In the following studies the coupling of the diaziridine to cells or plasma membranes was induced by a single flash at room temperature. In the dark, [^{3}H]cyclosporin diaziridine enters isolated rat-liver parenchymal cells. Equilibrium is reached after 5 min (Fig. 1). We conclude that the diaziridine derivative is recognized by cell structures as is cyclosporin A (data will be published in a separate paper).

Photoaffinity labeling of isolated rat-liver plasma membranes

Isolated plasma membranes were treated with one flash in the absence or presence of [^{3}H]cyclosporin-diaziridine. This procedure did not destroy membrane proteins (identical polypeptide patterns in SDS gels before and after photolysis) [21]. The concentrations of [^{3}H]cyclosporin diaziridine used in our experiments did not alter the polypeptide pattern of isolated rat-liver membranes. Labeling of specific proteins was characterized by SDS-polyacrylamide gel electrophoresis. The ^{3}H-labeled polypeptides were identified by fluorography. The radioactivity bound to the polypeptides was quantitated in sliced gel rods. Ten percent of the applied label (0.1–2 μM label, 3 min preincubation, one ultraviolet light flash at room temperature, 0.3 mg protein) remained asso-

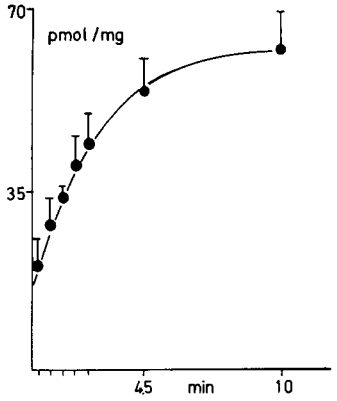
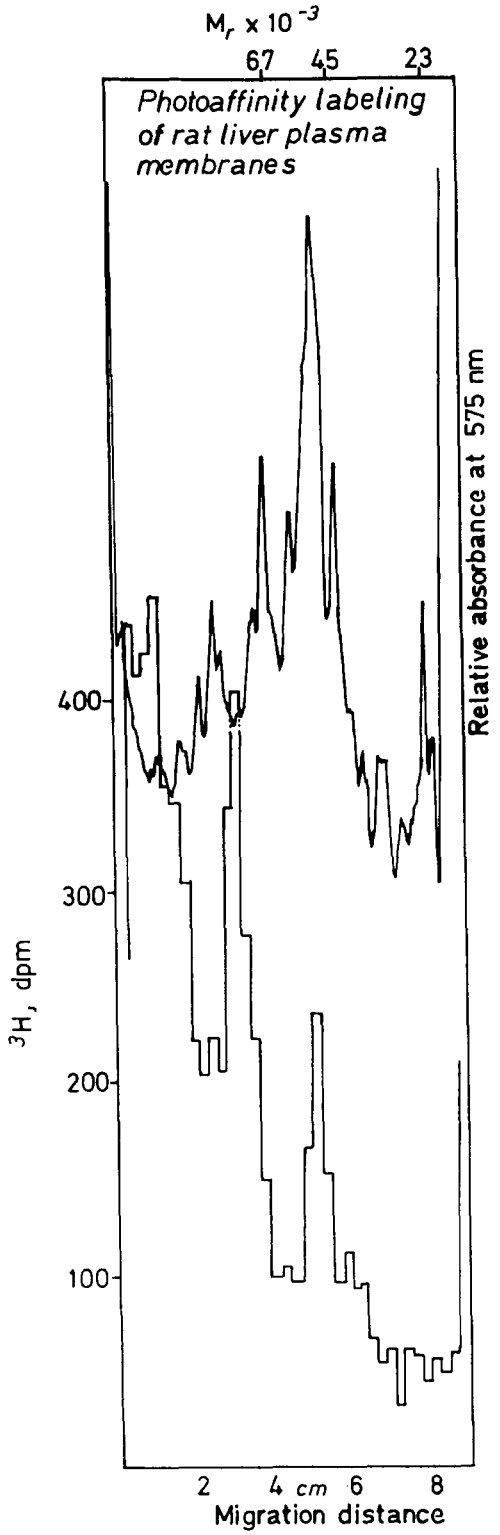


Fig. 1. [^{3}H]Cyclosporin-diaziridine uptake by isolated hepatocytes in the dark. Isolated hepatocytes ($2 \cdot 10^6$/ml in Tyrode buffer (pH 7.4) were incubated at 37°C in the dark with 400 pmol [^{3}H]cyclosporin-diaziridine per ml. At the times indicated 100 μl of cell suspension were withdrawn and uptake was measured by centrifugation through silicon oil as described in Ref. 24. The uptake per mg of cell protein was estimated. Shown are the means of three independent experiments.



ciated with several membrane proteins after electrophoresis. 18% of the label associated with membrane proteins was bound to a 85 kDa (±5000) protein, 5% of the label to a 50 kDa protein, in most of the experiments. Additional proteins with molecular mass of 200, 54 and 34 kDa are modified in 80% of the gels. A further protein with 140 kDa was also seen (Fig. 2). The principally labeled 85 kDa polypeptide represents a minor protein in silver-stained SDS gels and contains 2% of the total protein applied to the gel. In contrast, the 50 kDa protein amounts to 10% of the total protein. The incorporation of [^{3}H]-cyclosporin diaziridine into the 200, 85, 54, 50, 34 kDa proteins was light-dependent. When the photolabel and the membranes were mixed and placed in the dark for 1–15 min, background labeling occurred without the typical peak profile. The same phenomenon was seen after addition of prephotolysed [^{3}H]cyclosporin diaziridine to membranes, (time lag between photolysis and addition to the membrane 1 min), demonstrating that no long-living photoproducts exist (data not shown). To rule out the possibility that the regions of high specific incorporation are due to interligand insertions or the reaction of the photoprobe with the buffer, prephotolysed [^{3}H]cyclosporin-diaziridine medium samples were electrophoresed without membrane proteins; no peak profile was observed. Binding of photolysed [^{3}H]cyclosporin-diaziridine to the 200, 85, 50, 34 kDa polypeptides approaches saturation at concentrations of 2 μM (Fig. 3). Binding of [^{3}H]cyclosporin-diaziridine to membrane proteins can be prevented by simultaneous incubation with cyclosporin A, indicating the identity of the binding sites for cyclosporin and its diaziridine-derivative. Furthermore, mem-

Fig. 2. Photoaffinity labeling of isolated rat liver plasma membranes with [^{3}H]cyclosporin-diaziridine. Isolated rat liver plasma membranes (0.3 mg/ml) in phosphate-buffered saline (pH 7.4) were preincubated 3 min in the dark at room temperature with 0.4 μM of label. Photolysis was induced by a single ultraviolet light flash. Membranes were washed to remove unbound label. SDS solubilized plasma membrane proteins were separated by SDS gel electrophoresis. The distribution of radioactivity was determined by slicing polyacrylamide gel rods. Proteins were stained by Coomassie blue and scanned at 575 nm. Upper curve; Protein pattern, lower curve: radioactivity pattern.

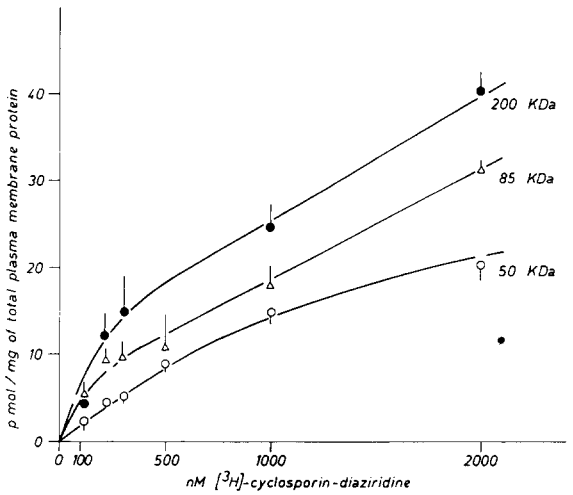


Fig. 3. Saturation of photoaffinity labeling of isolated rat liver plasma membranes. Isolated rat liver plasma membranes were preincubated 3 min in the dark with 0.1–2 μM [^{3}H]cyclosporin-diaziridine before one single ultraviolet flash. After washing, the membranes were separated by SDS gel electrophoresis. The radioactivity bound to the 200, 85, 50 kDa protein was determined by slicing gel rods. The radioactivity bound to the polypeptides is plotted versus nM of [^{3}H]cyclosporin-diaziridine (mean $\pm$ S.D., $n = 3$).

brane proteins can be protected against binding of [^{3}H]cyclosporin-diaziridine either by substrates of the bile-acid transporter in a concentration-dependent manner (100 and 500 μM of taurocholate, phalloidin [22], iopodate [23] Fig. 4) or by the affinity label isothiocyanatobenzamidocholate [9]. Comparison of the labeling pattern of [^{3}H]cyclosporin-diaziridine- and isothiocyanatobenzamido [^{3}H]cholate-labeled proteins reveals that cyclosporin binds to polypeptides with the same molecular mass (50, 34 kDa) susceptible to isothiocyanatobenzamidocholate, with the only exception being the 85 kDa protein (Fig. 5). Our protection studies show, however, that bile acids at higher concentrations (100–500 μM) interact with this protein too.

In experiments on isolated plasma membranes, the photoaffinity label may bind to both surfaces. Using intact cells one can expect preferential binding to the outside, if the incubation period is short enough. By comparison of labeled isolated membranes and whole cells we hope to see differences in radioactive protein pattern.

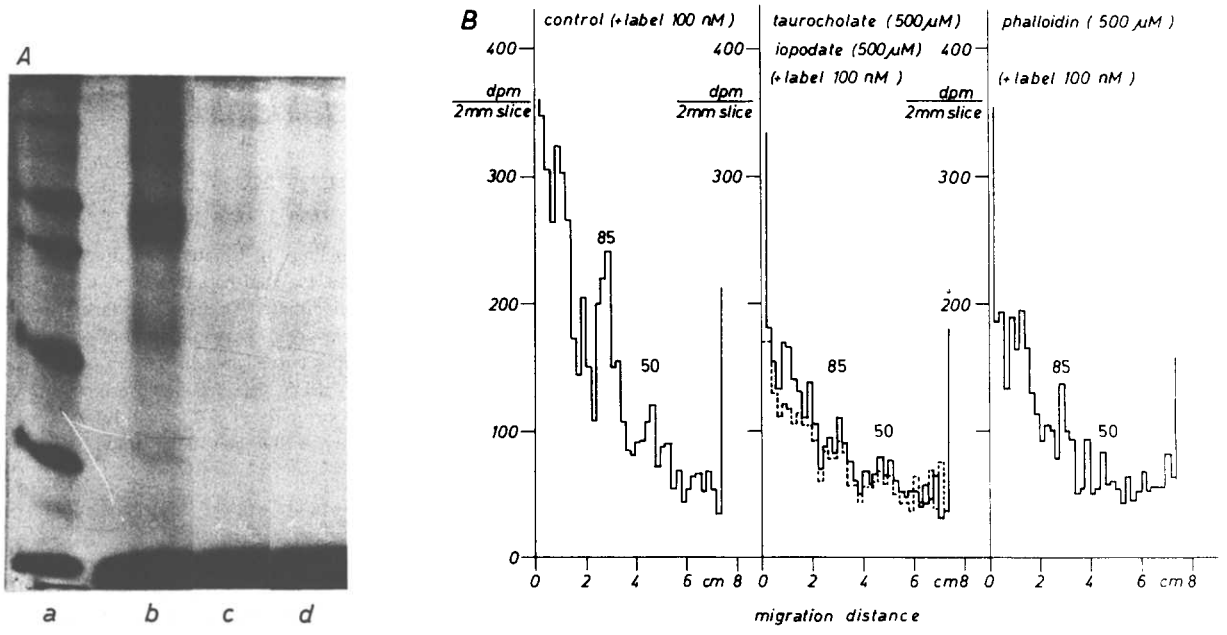


Fig. 4. Protection of isolated plasma membranes by cyclosporin A, isothiocyanatobenzamidocholate, taurocholate, iopodate and phalloidin against photoaffinity labeling with [^{3}H]cyclosporin-diaziridine. Isolated plasma membranes were preincubated without or with 500 μM protector and 100 nM label in the dark for 3 min before one single ultraviolet light flash. After washing, the membranes were separated by SDS gel electrophoresis. Shown is a fluorogram (A) and the distribution of radioactivity in sliced gel rods (B). A, Protein standard; Ab, control; Ac, cyclosporin A; Ad, isothiocyanatobenzamidocholate.

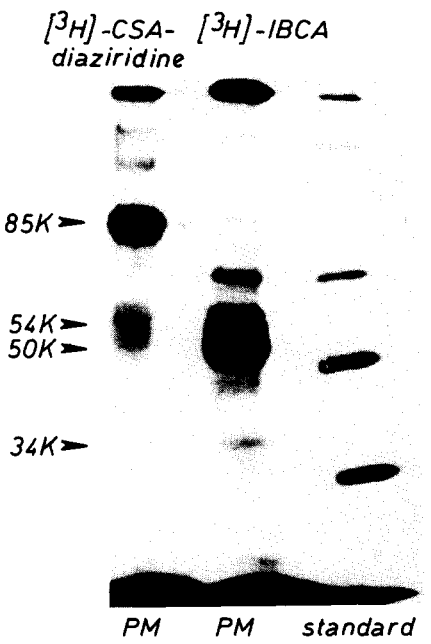


Fig. 5. Comparison of the labeling pattern of [^{3}H]cyclosporin-diaziridine and isothiocyanatobenzamido[^{3}H]cholate (photo) affinity-labeled rat liver plasma membranes. Isolated plasma membranes were photoaffinity labeled with [^{3}H]cyclosporin-diaziridine or affinity labeled with isothiocyanato-benzamido[^{3}H] cholate. Shown are fluorograms of SDS slab gels. Standard proteins: 200 kDa myosin, 92 kDa phosphorylase *b*, 69 kDa bovine serum albumin, 46 kDa ovalbumin, 30 kDa carbonic anhydrase, 14 kDa lysozyme. PM, plasma membranes: 1, [^{3}H]cyclosporin-diaziridine photolabeled proteins; 2, isothiocyanatobenzamido[^{3}H]cholate affinity-labeled proteins.

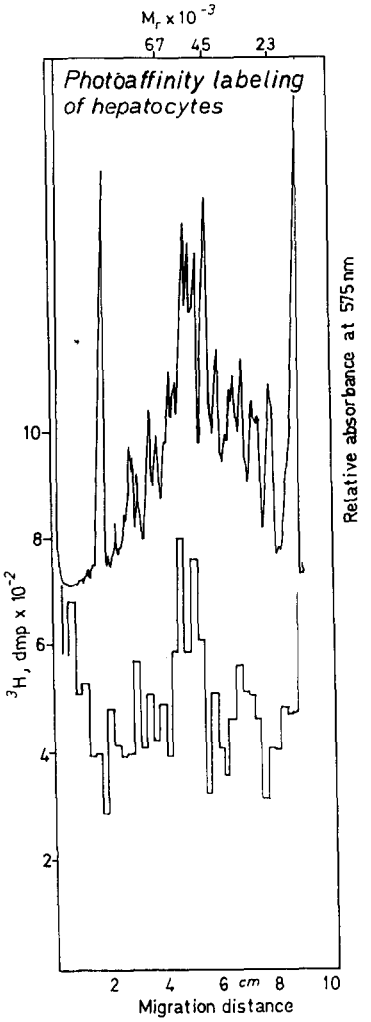


Fig. 6. Photoaffinity labeling of isolated hepatocytes with [^{3}H]cyclosporin-diaziridine. Isolated hepatocytes ($0.6 \cdot 10^6$/ml) in Tyrode buffer were preincubated 3 min in the dark at room temperature with [^{3}H]cyclosporin-diaziridine before one single ultraviolet light flash. Upper curve: protein pattern of isolated hepatocytes separated by SDS gel electrophoresis (Scan of Commassie blue stained proteins at 575 nm); lower curve: distribution of radioactivity in SDS gels, determined by slicing gel rods.

Photoaffinity labeling of isolated rat hepatocytes with [^{3}H]cyclosporin-diaziridine

The labeling pattern of isolated hepatocytes was distinct from that of isolated plasma membranes when using identical experimental conditions (room temperature, 3 min preincubation, one ultraviolet light flash). The 54 and 50 kDa polypeptides are the major labeled proteins (Fig. 6). Cyclosporin-diaziridine is taken up into hepatocytes in the dark; we therefore studied the time and temperature-dependent alteration in the [^{3}H]cyclosporin-diaziridine labeling pattern of isolated hepatocytes.

Isolated hepatocytes were preincubated in the dark with 5.6 μM of [^{3}H]cyclosporin-diaziridine for different periods (1, 3, 10, 30 min) and different temperatures (4, 10, 18, 22, 30°C) before photolysis with a single ultraviolet light flash at room temperature. Time as well as temperature dependent increase in the amount of bound label was observed. Furthermore, the pattern of modified proteins varies at different times and temperatures. At 0°C and short incubation time (up to 3 min) the 50 kDa protein was labeled exclusively.

At 30°C and after a longer incubation period (up to 30 min) the 85 kDa and the 34 kDa proteins became additionally labeled [21]. Between 18 and 22°C binding of [^{3}H]cyclosporin-diaziridine increased markedly (Fig. 7). Preincubation of hepatocytes for 1 min in the dark at 30°C and subsequent photolysis labeled the 50 kDa protein only. The 85 kDa and 34 kDa protein were modified additionally, when the preincubation period was markedly extended. Since [^{3}H]cyclosporin-diaziridine is taken up by hepatocytes we suggest that the 85 and 34 kDa protein may be localized at the cytoplasmic side of the hepatocellular membrane.

On cells disrupted by sonication, all proteins with affinity to cyclosporin diaziridine should be labeled after short preincubation. Photoaffinity labeling of sonicated hepatocytes produced a pattern of radioactivity different from that of intact cells and also of isolated plasma membranes. The 85 kDa protein is the major labeled protein in isolated plasma membranes. It was labeled in sonicated cells but not in intact cells, under the conditions used here (Fig. 8).

As described earlier, cyclosporin inhibits the uptake of bile acids and phalloidin [24]. The [^{3}H]cyclosporin-diaziridine labeling pattern of hepatocytes resembles that of isothiocyanatobenzamido[^{3}H]cholate. That means some membrane proteins modified by [^{3}H]cyclosporin-diaziridine may be involved in the transport of bile acids (or of phalloidin). It is of interest whether substrates of the bile-acid transporter protect these proteins against [^{3}H]cyclosporin-diaziridine labeling. The results are shown in Table I. The radioactivity bound to the above proteins is reduced in the presence of iopodate, phalloidin, cholate, DIDS and bromosulfophthalein [25]. It was, however, impossible to prevent [^{3}H]cyclosporin-diaziridine coupling totally. Further control experiments (dark control, interaction of cells with prephotolysed label) gave negative results (data not shown).

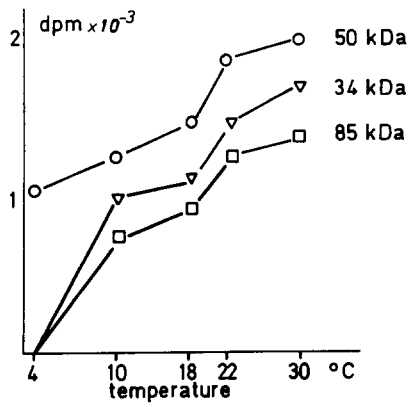


Fig. 7. Temperature dependence of photoaffinity labeling of isolated hepatocytes by [^{3}H]cyclosporin-diaziridine. Isolated hepatocytes were preincubated at 4, 10, 18, 22 and 30°C in the dark for 10 min with 5.6 μM of label before one single ultraviolet light flash. After extensive washing the cell proteins were separated by SDS gel electrophoresis. The radioactivity bound to the 50, 34, and 85 kDa polypeptides was estimated by slicing SDS gel rods.

Binding of [^{3}H]cyclosporin-diaziridine to cytosolic proteins

The existence of intracellular binding proteins for cyclosporin has been supposed. Such a protein (cyclophilin) as been isolated from T-lymphocytes, the main pharmacological target of cyclosporin A [26]. The molecular mass of cyclophilin from T-lymphocytes is 15 kDa. For detection of an identical or similar protein in liver cells we used a system with an acrylamide concentration of 15%: proteins down to 10 kDa can be identified. Cytosol from liver cells was isolated from [^{3}H]-cyclosporin-diaziridine photolabeled cells (prein-

TABLE I

PROTECTION OF ISOLATED HEPATOCYTES AGAINST PHOTOAFFINITY LABELING BY [^{3}H]CYCLOSPORIN DIAZIRIDINE

Isolated hepatocytes ($2 \cdot 10^6$/ml) were photoaffinity labeled with 5.6 μM [^{3}H]cyclosporin diaziridine in absence or presence of 500 μM phalloidin, cholate, iopodate, bromosulfophthalein (BSP) and 100 μM DIDS. The radioactivity bound to the 50 and 85 kDa protein was quantitated by slicing SDS gel rods. Shown is the percentage of inhibition of the control, mean ± S.D. ($n = 3$).

	% Inhibition of control	
	50 kDa	85 kDa
Phalloidin	30 ± 5.5	26 ± 20
Cholate	24 ± 8	34 ± 12
Iopodate	30 ± 15	33 ± 20
BSP	25 ± 3.6	34 ± 20
DIDS (100 μM)	45 ± 10	68 ± 12

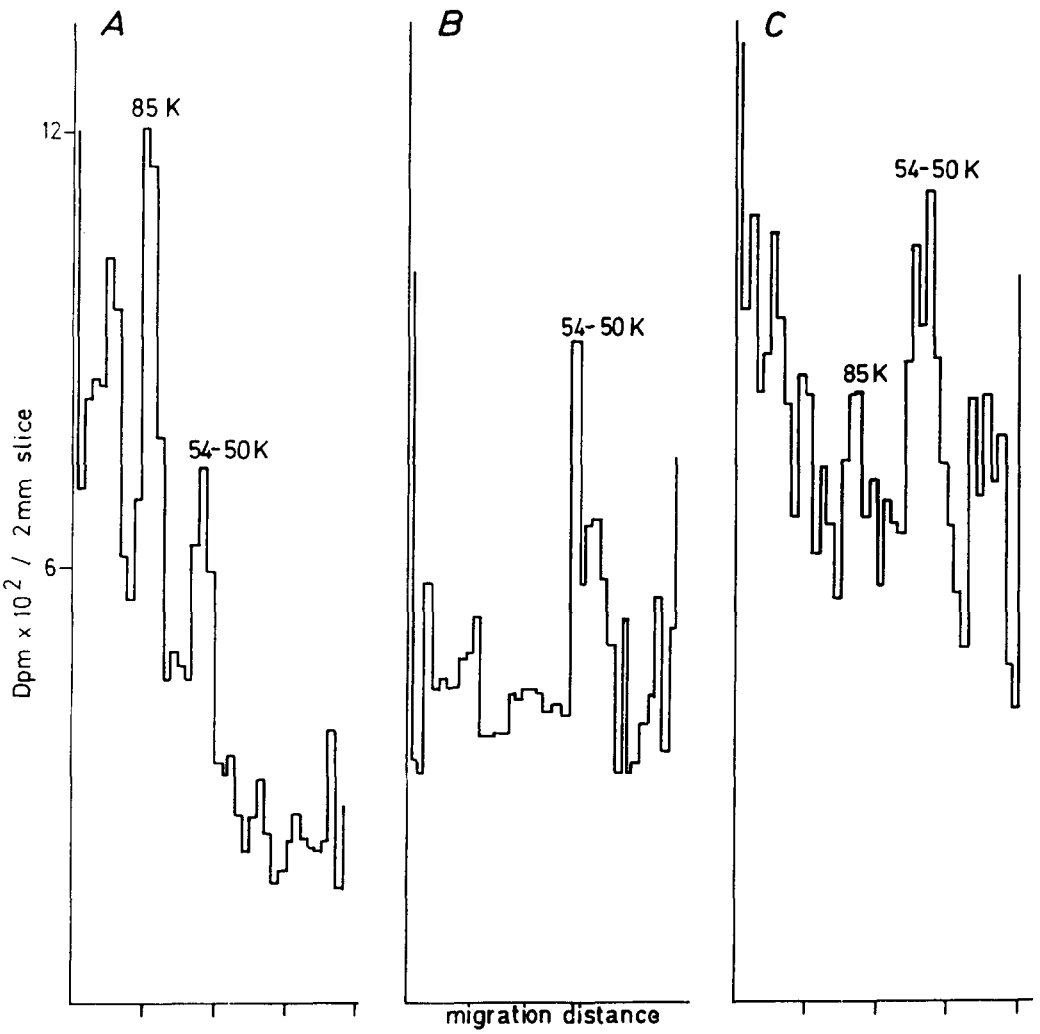


Fig. 8. Photoaffinity labeling of isolated plasma membranes, isolated intact hepatocytes and disrupted hepatocytes by [^{3}H]cyclosporin-diaziridine. Isolated hepatocytes ($0.6 \cdot 10^6$/ml), isolated rat liver plasma membranes (0.3 mg/ml) or disrupted liver cells (by sonication) were preincubated 3 min at room temperature in the dark with 5.6 μM of [^{3}H]cyclosporin-diaziridine. After a single ultraviolet light flash, the protein of the hepatocellular membranes were separated by SDS polyacrylamide gel electrophoresis. The radioactivity bound to the polypeptides was quantitated by slicing gel rods. (A) Plasma membranes; (B) intact hepatocytes; (C) disrupted hepatocytes.

cubation in the dark for 30 min). Labeled proteins with molecular mass of 22 and 17 kDa were identified. (Data not shown).

Photoaffinity labeling of rat AS-30D ascites hepatoma cells and Ehrlich ascites cells

AS-30D ascites hepatoma cells and Ehrlich ascites cells are known to be unable to transport bile acids [13]. Therefore labeling with an affinity label derived from cholate resulted in background labeling only [9]. Photoaffinity labeling with [^{3}H]cyclosporin-diaziridine modifies proteins with molecular mass of 200, 150, 80, 70, 42, 25 kDa in Ehrlich ascites tumor cells. The 200 kDa polypeptide was the major labeled protein. In AS-30D ascites hepatoma cells proteins with molecular mass of 90, 56, 30, 24, 20 kDa were labeled (90 kDa most heavily labeled) by cyclosporin. The function of these proteins is unknown (Fig. 9).

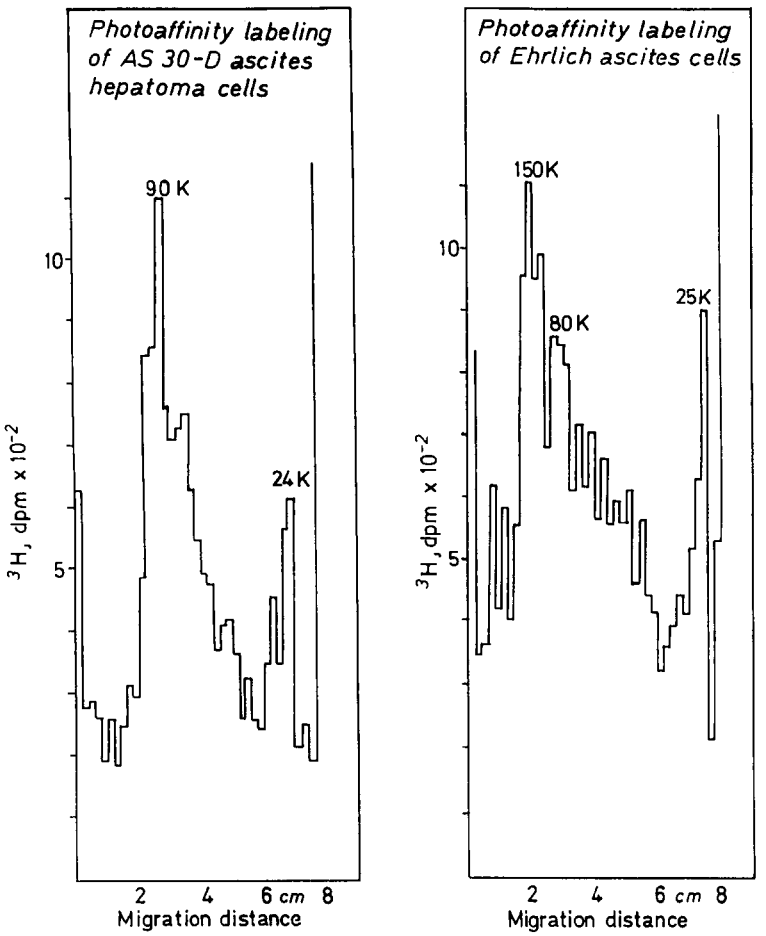


Fig. 9. Photoaffinity labeling of AS-30D ascites and Ehrlich ascites cells by [^{3}H]cyclosporin-diaziridine. AS-30D ascites and Ehrlich ascites cells ($10 \cdot 10^6$/ml) were preincubated with the label (5.6 μM) for 3 min at room temperature in the dark. Photolysis was induced by a single ultraviolet light flash. Cells were washed and the protein separated by SDS gel electrophoresis. The distribution of radioactivity was determined by slicing gel rods. (A) AS-30D ascites hepatoma cells; (B) Ehrlich ascites cells.

Discussion

In a previous study it was shown that cyclosporin inhibits the uptake of bile acids and of rifampicin (a drug transported by the bilirubin carrier [27]) in isolated hepatocytes [24]. [^{3}H]Cyclosporin-diaziridine, a derivative which can be photoactivated, was used to identify binding proteins for cyclosporin, in particular structures needed for the uptake of cyclosporin by hepatocytes. Cyclosporin-diaziridine fulfills the criteria for a true photoaffinity label. In the dark no coupling with proteins occurred. The hydrophobicity of cyclosporin-diaziridine led to background labeling only. Labeling did not occur when prephotolysed label was mixed with plasma membranes. This ruled out the existence of long-lived photoproducts. Photolabeling of specific proteins showed saturation kinetics. The original cyclosporin A protected proteins against photolabeling demonstrating the identity of binding sites. Furthermore, the labeling could be prevented by bile acids and substrates of the bile-acid carrier, and also by isothiocyanatobenzamidocholate. The labeling pattern of [^{3}H]cyclosporin-diaziridine or isothiocyanatobenzamido[^{3}H] cholate was identical, one exception being the 85 kDa protein. The interaction, however, of bile acids with the 85 kDa protein became apparent by protection studies using bile-acid concentrations up to 500 μM. Kinetic studies reported earlier, led to the suggestion that cyclosporin binds to the bile-acid transporter without being transported itself. This conclusion agrees with the results of the labeling studies. The 85 kDa protein was heavily labeled. It had the highest affinity to cyclosporin A in iso-

lated plasma membranes and represented 2% of the total protein only. This protein was not modified in whole cells at 0°C and after a preincubation of 3 min in the dark. Under those conditions the 50 kDa proteins was the major labeled polypeptide. At 0°C cyclosporin was not taken up by hepatocytes [28]. At temperatures above 10°C cyclosporin was successively incorporated into hepatocytes, and the 85 kDa protein could be identifified. This suggests a localisation at the cytoplasmic side of the hepatocellular membrane. The uptake of cyclosporin A was independent of the energy supply. On the other hand, intracellular accumulation occurred [28]. Intracellular binding proteins may account for this concentrative uptake. A cytosolic binding protein for cyclosporin had been isolated from T-lymphocytes (15 kDa [26]). In the cytosol of rat liver cells, [^{3}H]-cyclosporin diaziridine coupled with a 22 and a 17 kDa protein. Apparently hepatocytes also contain cytosolic binding proteins.

Cyclosporin inhibits the uptake of bile acids into liver cells in a non-competitive manner [24]. The bile-acid transporter seems not to be involved in the uptake of cyclosporin. This heterospecific transport system [29] is only present in liver cells, but not in hepatoma or Ehrlich ascites cells. The lack of transport function corresponds to the results obtained after labeling with isothiocyanatobenzamido[^{3}H]cholate [9]. Affinity labeling with isothiocyanatobenzamido[^{3}H]cholate results in background labeling only. In contrast cyclosporin coupled with several proteins, among them, polypeptides with molecular mass of 85 ($\pm$5000) and 200 kDa. The function and properties of these proteins, especially the 85 kDa protein, will be further studied.

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