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Cyclosporin A and a diaziridine derivative inhibit the hepatocellular uptake of cholate, phalloidin and rifampicin

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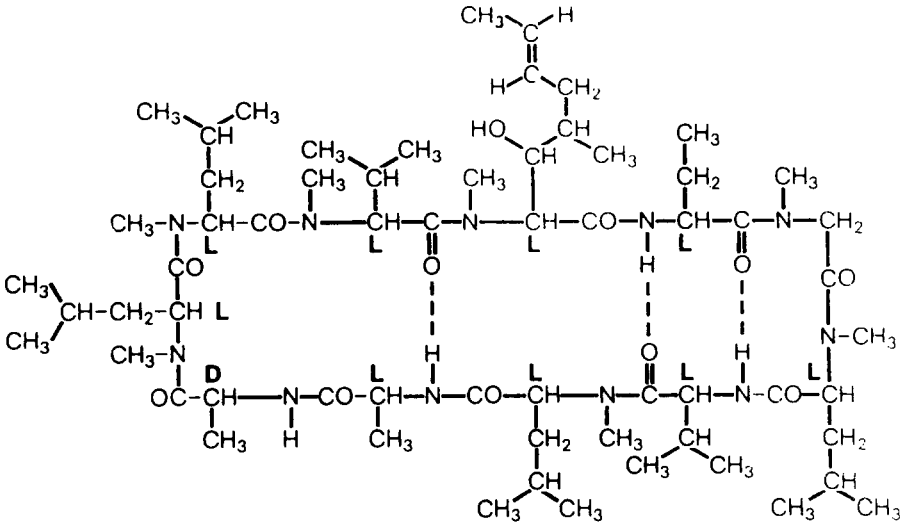
Cyclosporin A inhibits the uptake of cholate into isolated hepatocytes in a non-competitive manner ($K_i = 3.6$ μM). It protects liver cells against phalloidin injury by a mixed competitive/non-competitive inhibition of phalloidin uptake ($K_i = 0.08$ μM). Rifampicin, a well-known substrate of the bilirubin transporter is also incorporated in a decreased quantity in the presence of cyclosporin A ($IC_{50} = 80$ μM). A photolabile diaziridine derivative of cyclosporin A was used for the identification of binding sites. In comparison with the original cyclosporin A the photoaffinity label exhibits a 2–3-fold lower affinity to the cholate (and phalloidin) transporter in the liver cell membrane. In the dark the label inhibits the uptake of both cholate and of phalloidin reversibly; after treatment with ultraviolet light flashes the inhibition becomes irreversible. The degree of inhibition is concentration dependent. Our results suggest binding of cyclosporin A to protein components of the cholate (and phalloidin) transporter of liver cells without uptake by this system. The inhibition of cholate (and phalloidin) uptake by cyclosporin A is non-competitive and may be due to nonspecific hydrophobic binding to compounds of the cholate transporter.

Introduction

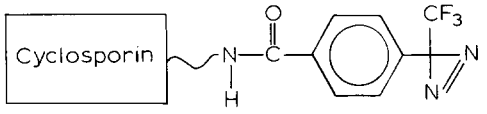
Cyclosporin A, a cyclic endecapeptide, with potent immunosuppressive properties [1], is extensively metabolized after accumulation in the liver [2,3]. The major route of elimination of cyclosporin A and its metabolites is biliary excretion [2]. Accordingly, hepatotoxicity is one of the side effects of cyclosporin A treatment. Plasma bile salt and direct plasma bilirubin levels are increased in cyclosporin A-treated patients (cholestasis). However, significant alterations in enzyme levels were not found [4,5].

In a previous paper [6] we proposed that cholestasis might be caused by competition between the uptake of cyclosporin A and bile acids. On the other hand, cyclosporin A was found to protect isolated hepatocytes against phalloidin poisoning [6]. Phalloidin, a bicyclic heptapeptide is one of several foreign compounds which can be transported by (one of) the bile acid carriers (multispecific transport system) [7,9]. Because of the peptide structure of cyclosporin we speculated that cyclosporin might be a further candidate for transport by the above system. However, this multispecific system is not responsible for the hepatocellular uptake of all organic anions. Bilirubin, bromosulfophthalein and rifampicin are known to be taken up by another transporter [10]. Whereas the cholate transport system is a secundary active one, the bilirubin system works by facilitated diffusion. It is of interest whether cyclosporin impaires the latter system or not. Other date suggest that the drug interacts with a series of membrane proteins [11]. The following studies demonstrate uptake kinetics and binding experiments with a

Cyclosporin A



Cyclosporin - diaziridine



photolabile diaziridine derivative of cyclosporin A indicative for interaction of cyclosporin A with both bile acid and bilirubin transporting system. In contrast the translocation of aminoisobutyric acid was unaffected by cyclosporin in Ehrlich ascites tumor cells.

Materials

Cyclosporin A and cyclosporin diaziridine were gifts from Dr. R. Wenger (Sandoz AG, Basel, Switzerland). 2-Amino[^{14}C]isobutyric acid (spec. act. 58 mCi/mmol), [^{14}C]cholate (spec. act. 52 mCi/mmol) were purchased from Amersham Buchler GmbH, Braunschweig, F.R.G. Demethyl[^{3}H]phalloin (spec. act. 3.5 Ci/mmol) was a gift from Dr. T. Wieland and Dr. H. Faulstich, Heidelberg, F.R.G.; phalloidin from Dr. Madaus, Köln, F.R.G.; [^{14}C]Rimactan (spec. act. 3.9 μCi/μmol) from Ciba, Basel, Switzerland. Collagenase was purchased from Boehringer, Mannheim, F.R.G. All other materials used were of reagent grade quality.

Methods

Preparation of hepatocytes

Hepatocytes were isolated according to the method of Berry and Friend [12] by perfusion with 0.05% collagenase, in a Krebs-Henseleit buffer. After an equilibration period of 30 min at 37°C in O_2/CO_2 (95 : 5, v/v) atmosphere in Tyrode buffer (pH 7.4) 85–90% of the isolated cells were viable as judged by Trypan blue exclusion. All experiments were performed within 2 h after cell isolation.

Protein was determined by the biuret method using bovine serum albumin as the standard.

Uptake studies

Uptake of phalloidin and of cholate was measured by rapid centrifugation of radioactive cell suspensions through silicon oil [13]. Uptake measurements were started by the addition of a mixture of either demethyl[^{3}H]phalloin and phalloidin or [^{14}C]cholate and cholate at a final concentration of 0.1 and 6 μM or 1 and 6 μM, respectively. Aliquots (100 μl) were withdrawn at the indicated times and centrifuged through silicon oil. Thereby the reaction was stopped within 1–3 s. The radioactivity associated with the cells was measured in Lipoluma/Lumasolve/water (100 : 10 : 2, v/v). The extracellular fluid trapped in the cellular pellet was quantitated by addition of [^{3}H]inulin. The calculated inulin space was 0.5 μl/mg protein. No corrections were made for the determination of the initial uptake rate for cholate of 15–165 s, since the inulin space was constant during this period.

Uptake of rifampicin and aminoisobutyric acid was measured using 10 μM [^{14}C]rimactan per $2 \cdot 10^6$ hepatocytes/ml, or 10 μM amino[^{14}C]isobutyric acid plus 2 mM aminoisobutyric acid.

Studies on uptake kinetics

To determine the type of phallotoxin and cholate uptake inhibition by cyclosporin A, the following studies were performed: 1 ml of isolated hepatocytes was incubated with 0.8, 4, 8, or 0.04, 0.08, 0.4 μM cyclosporin A for 30 s prior to the addition of a constant concentration of [^{14}C]-cholate or demethyl[^{3}H]phalloin and varied concentrations of unlabeled substrates. To avoid the influence of cell aging on the uptake kinetics, the measurements were done simultaneously using the same cell preparation. The initial rate of uptake was calculated from the slope of the uptake curve. These data were plotted according to Lineweaver and Burk [14]. K_i was determined according to Dixon [15].

Reversible inhibition of uptake by cyclosporin diaziridine in the dark

Reversible inhibition of cholate and phallotoxin by cyclosporin-diaziridine was tested by incubation of hepatocytes with varied concentrations of the inhibitor in the dark. Uptake measurements were started 30 s after addition of the derivative.

Inhibition of phalloidin and cholate uptake by cyclosporin diaziridine after photolysis with ultraviolet light flash

Covalent coupling of the photosensitive cyclosporin derivative to hepatocytes was induced by a single ultraviolet light flash with a duration of one millisecond (the design of the apparatus will be described in a separate report). Hepatocytes were incubated with varied concentrations of cyclosporin diaziridine at 37°C in O_2/CO_2 atmosphere for 3 min before photolysis by a single ultraviolet light flash. Thereafter, the cells were washed extensively and the uptake of phalloidin and cholate was measured.

Quantitative analysis of phalloidin effects in isolated hepatocytes

The inhibition by cyclosporin A and cyclosporin diaziridine of a typical phalloidin effect was tested on isolated hepatocytes at 37°C. Hepatocytes develop characteristic blebs in the presence of phalloidin [16]. The ratio of affected to non-affected cells depends on the concentration of the toxin. Varied concentrations of cyclosporin A or cyclosporin diaziridine were incubated with 1 ml of cell suspension ($2 \cdot 10^6$/ml) 1 min before addition of phalloidin at a final concentration of 10 μM. The strength of the phalloidin response in isolated hepatocytes was expressed as the percentage of cells affected by 10 μM phalloidin within 20 min.

Results

Inhibition of cholate and phallotoxin transport by cyclosporin A

Cyclosporin A inhibits cholate uptake in a non-competitive manner as can be seen by the common intercept at the abscissa in the Lineweaver-Burk plot (Fig. 1A). The inhibitor constant K_i was calculated by plotting the reciprocal velocity of the uptake versus the inhibitor concentration, according to Dixon (Fig. 2A). The K_i for cholate-transport inhibition was calculated to be 3.6 μM. The shape of the Dixon plot is consistent with non-competitive inhibition. In contrast, phal-

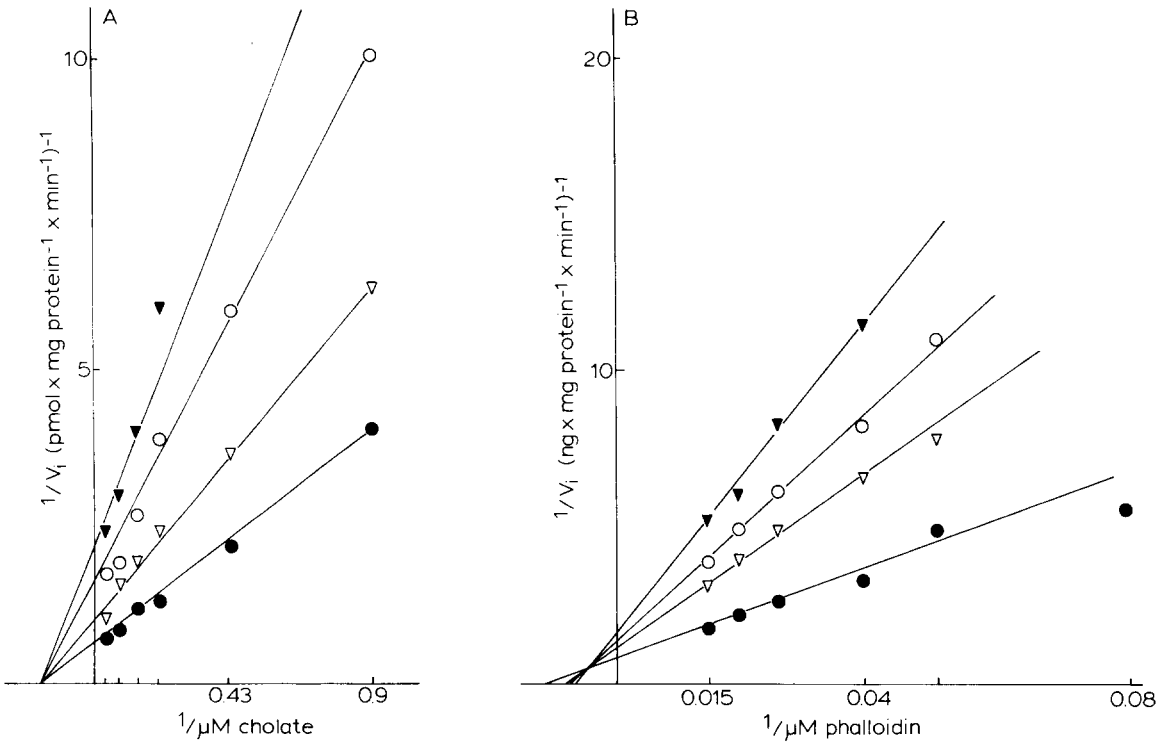


Fig. 1. (A)Lineweaver-Burk plot of the uptake of cholate in the presence of cyclosporin A. Isolated hepatocytes ($2 \cdot 10^6$/ml) were incubated with 0.8 (▽———▽), 4 (○———○), and 8 (▼———▼) μM of cyclosporin A 30 s prior to the addition of a mixture of 1 μM [^{14}C]cholate with 11–230 μM of cholate. The rate of uptake (V_i) was calculated within the first 3 min after addition of cholate ($n = 4$). ●———●, control. (B) Lineweaver-Burk plot of the uptake of phalloidin in the presence of cyclosporin A. Isolated hepatocytes were incubated with 0.04 (▽———▽), 0.08 (○———○), 0.4 (▼———▼) μM of cyclosporin A 30 s prior to the addition of a mixture of 0.1 μM demethyl[^{3}H]phalloin with 19–63 μM phalloidin. The rate of uptake (V_i) was calculated within the first 5 min after addition of phalloidin ($n = 5$). ●———●, control.

lotoxin transport is inhibited by cyclosporin A in a mixed competitive and non-competitive manner (common intercept above the abscissa). The K_T as well as V_{max} are altered (Fig. 1B). The K_i was calculated to be 0.08 μM (Fig. 2B).

Inhibition of rifampicin uptake by cyclosporin A

Treatment of patients with cyclosporin A leads, in some cases, to increased plasma bilirubin levels [4]. The latter is taken up into hepatocytes by carrier mediated facilitated diffusion [10]. There is evidence that rifampicin (mainly eliminated by biliary excretion), is transported into hepatocytes via the bilirubin transporting system (Petzinger, E., unpublished data). Rifampicin inhibits the biliary excretion of bilirubin and also the uptake of bromosulfophthalein by isolated hepatocytes [17]. Bromosulfophthalein, bilirubin, and rifampicin have been suggested to be transported into liver cells by means of the same carrier system [18]. We therefore studied the effect of cyclosporin A on the uptake of [^{14}C]rifampicin.

Cyclosporin inhibits the uptake of rifampicin in a concentration-dependent manner (Fig. 3). 50% inhibition of rifampicin uptake is achieved by 80 nmoles of cyclosporin A per millilitre of rat-liver cell suspension.

Lack of effect of cyclosporin A on the uptake of aminoisobutyric acid by Ehrlich ascites tumor cells

Whereas cyclosporin A inhibits the uptake of cholate, phalloidin and rifampicin by isolated hepatocytes, the Na^+-dependent active influx of aminoisobutyric acid by Ehrlich ascites tumor cells [19,20] was not inhibited at concentrations effective at the bile acid carrier (data not shown).

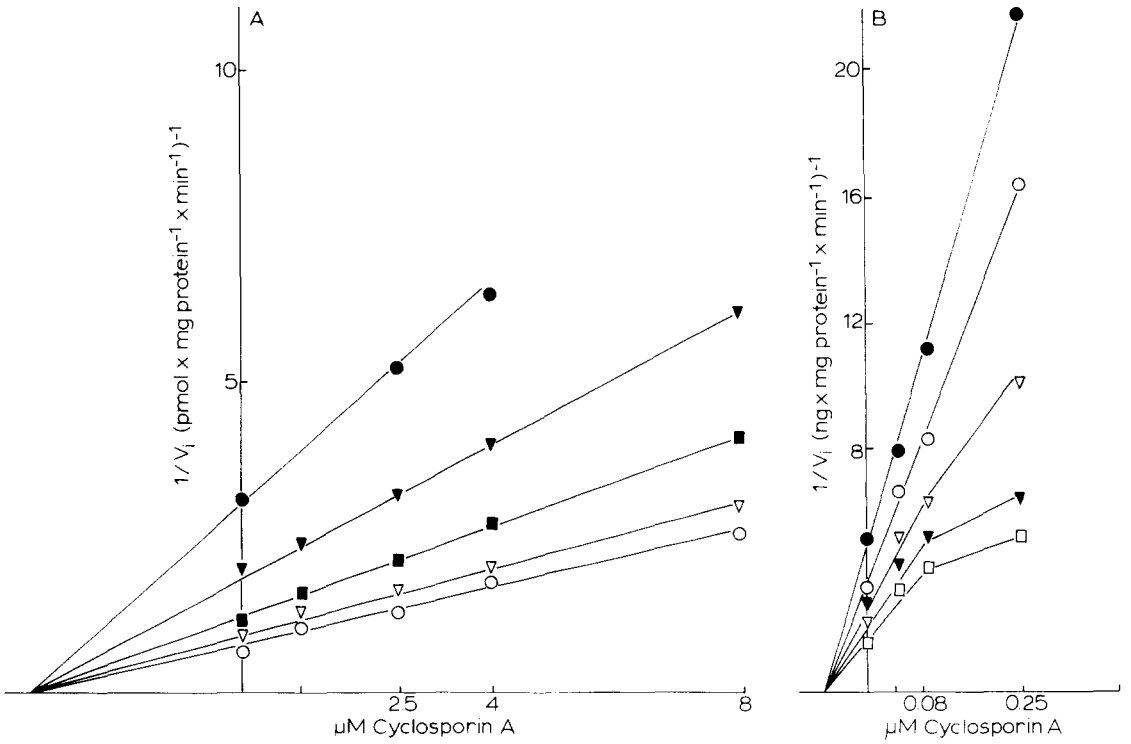


Fig. 2. (A) Dixon plot on the inhibition of cyclosporin A on the uptake of cholate. Isolated hepatocytes were incubated with 0.8, 2.5, 4, 8 μM of cyclosporin A 30 s prior to the addition of a mixture of 1 μM [^{14}C]cholate with 23 (●———●); 46 (▼———▼); 69 (■———■); 115 (▽———▽); 230 (○———○) μM of cholate. The initial rate of uptake (V_i) was calculated (n = 3). (B) Dixon plot of the inhibition of cyclosporin A on the uptake of phalloidin. Isolated hepatocytes were incubated with 0.04, 0.08, 0.25 μM of cyclosporin A 30 s prior to the addition of a mixture of 0.1 μM demethyl[^{3}H]phalloin with 19 (●———●), 25 (○———○), 38 (▽———▽), 50 (▼———▼), 60 (□———□) μM phalloidin. (n = 4).

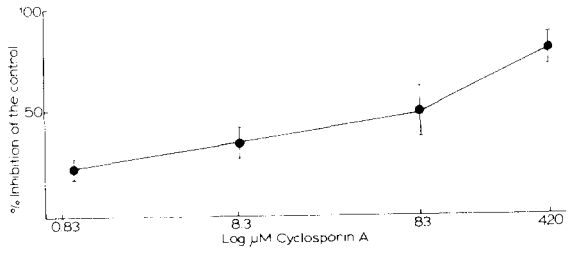


Fig. 3. Inhibition of rifampicin uptake by cyclosporin A. Isolated hepatocytes ($2 \cdot 10^6$/ml Tyrode buffer) were incubated with varied concentrations of cyclosporin A 30 s prior to the addition of rifampicin at a final concentration of 10 μM [^{14}C]rifampicin. The initial uptake was measured. The inhibition of V_i rifampicin uptake by cyclosporin A is plotted versus the log cyclosporin concentration (μM). Shown are the means of four different experiments.

Inhibition of phallotoxin response by cyclosporin A and cyclosporin diaziridine

Cyclosporin diaziridine, a photolabile derivative of cyclosporin A, protects isolated hepatocytes against phalloidin poisoning. The affinity to the phalloidin transport system is one third that of the original cyclosporin A. 50% inhibition of phalloidin response is achieved by 100 μM cyclosporin A or 300 μM cyclosporin diaziridine (Table I).

Inhibition of cholate and phalloidin transport by cyclosporin A and cyclosporin diaziridine in the dark

Cyclosporin A and its diaziridine derivative inhibit both phallotoxin and cholate transport in isolated hepatocytes. Cyclosporin A exhibits higher affinity to the transport system than the diaziridine derivative. Phallotoxin and cholate uptake is 50% inhibited by, respectively, 240 nM and 6000

TABLE I

REVERSIBLE INHIBITION BY CYCLOSPORIN A AND CYCLOSPORIN DIAZIRIDINE OF CHOLATE AND PHALLOTOXIN UPTAKE AS WELL AS PHALLOIDIN RESPONSE IN ISOLATED HEPATOCYTES

Isolated hepatocytes ($2 \cdot 10^6$/ml) in tyrode buffer (pH 7.4 at 37°C) were incubated 30 s with varying concentrations of cyclosporin A or cyclosporin-diaziridine (in the dark). Uptake measurements were started by addition of a mixture of 0.1 μM demethyl[^{3}H]phalloin plus 6 μM phalloidin or 1 μM [^{14}C]cholate plus 6 μM cholate (final concentrations). The inhibition of the initial uptake (V_i) was determined ($n = 4$). The inhibition of the phalloidin response was evaluated by preincubation of cyclosporin A or of the diaziridine derivative (1–3000 nM) for 1 min with $2 \cdot 10^6$ hepatocytes per millilitre. Phalloidin at a final concentration of 10 μM was added. After 20 min the percentage of cells showing protrusions was determined. The concentration needed to produce 50% inhibition of the control is given as mean ± S.D. ($N = 4$).

	50% reversible inhibition of		
	cholate transport (nM)	phallotoxin transport (nM)	phalloidin response (nM)
Cyclosporin A	3000 ± 30	90 ± 10	100 ± 20
Cyclosporin diaziridine	6000 ± 30	240 ± 15	300 ± 15

nM of cyclosporin diaziridine. In contrast, 90 nM or 3000 nM of cyclosporin A are needed to reduce uptake of phallotoxin or cholate by 50% (Table I).

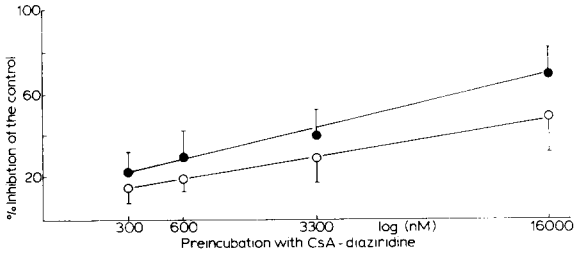


Fig. 4. Irreversible inhibition of phallotoxin and cholate uptake by cyclosporin-diaziridine. Isolated hepatocytes were preincubated 3 min at 37°C with 300, 600, 1600 nM of cyclosporin-diaziridine in the dark. Photolysis of the diaziridine derivative was induced by a single high-energy ultraviolet light flash of 1 ms at 25°C. Cells were washed in Tyrode buffer (pH 7.4) to remove unbound cyclosporin. Thereafter uptake of cholate [○——○] and phalloidin [●——●] were measured. The inhibition of the initial uptake (V_i) was determined. Shown are the means of four independent experiments.

Irreversible inhibition of cholate and phallotoxin transport by cyclosporin diaziridine after photolysis

Diaziridine produce coupling carbenes during photolysis. Our cyclosporin diaziridine was photolysed by a single high-energy ultraviolet light flash of 1 ms.

As a control, hepatocytes were exposed to light flash in the absence of the diaziridine derivative. Transport activity was only slightly impaired by this procedure. Irreversible inhibition of both cholate and phallotoxin uptake was observed by treatment of hepatocytes in the presence of cyclosporin diaziridine (Fig. 4). The concentrations needed to produce a 50% irreversible inhibition of cholate or phallotoxin uptake were 2.6- or 13-fold higher, respectively, than those for reversible inhibition.

Discussion

In a previous paper [6] we speculated that cyclosporin A might be incorporated as is phalloidin [7,9] by one of the bile salt transporters. The data presented now contradict this fascinating explanation of clinical [21,22] and experimental findings [6].

The noncompetitive inhibition by cyclosporin A of bile acid uptake is inconsistent with the above speculation. On the other hand cyclosporin seems to bind to membrane proteins undoubtly involved in the hepatocellular uptake of both cholate and phalloidin. It must be noted that cyclosporin A is a very hydrophobic peptide so that hydrophobic interactions with various proteins are probable. Another approach is the possibility of integration of cyclosporin A into the lipid bilayer, so that secundary impairment of protein functions become possible. In earlier studies with phalloidin we showed that its uptake by liver cells depends markedly on actual conditions in the lipid phase surrounding the transport protein. The presence of low amounts of phospholipase A, decrease of temperature below 20°C or loading of membranes with sylibin decreased the uptake of phalloidin drastically [23,24].

The best approach available at present is the assumption of modifier sites on the bile acid transporter similar to those described for the anion transporter of red cells [25,26]. It is of interest that

the bilirubin transporter (uptake of rifampicin), known to be different from the above system, is also inhibited by cyclosporin A. In contrast to cholate and phalloidin, bilirubin is taken up by facilitated diffusion. The affinity of cyclosporin A to the bilirubin system (examined with rifampicin) is lower than to the cholate system (50% inhibition of cholate, phalloidin and rifampicin uptake at substrate to inhibitor concentrations of 1 : 0.5, 1 : 0.015, 1 : 6.9, respectively). Since the uptake of [^{3}H]cyclosporin A (data will be published in a separate paper) is not inhibited by bilirubin or rifampicin it is highly improbable that the bilirubin system incorporates cyclosporin A into hepatocytes (evaluation of kinetics therefore without interest).

The reader might suspect that cyclosporin A intereacts with all membrane proteins in a non-specific manner. We tested therefore the Na^+-dependent uptake of aminoisobutyric acid into Ehrlich ascites tumor cells in presence of cyclosporin A, but without any inhibition by the latter.

The properties of the diaziridine label have to be discussed. The introduction of the diaziridine moiety reduces the affinity of cyclosporin to the bile salt transporter to 30–50%. In addition the concentration of the photoaffinity label needed for 50% irreversible inhibition of the cholate (and phalloidin) uptake by hepatocytes are 2.6 (and 13)-fold higher as measured in the dark (reversible inhibition). Nevertheless [^{3}H]cyclosporin diaziridine is a suitable label for examination of cyclosporin binding proteins in biological objects.

Acknowledgements

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