

Mechanisms of Hepatic Transport of Cyclosporin A: An Explanation for its Cholestatic Action?

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The hepatic transport of the immunosuppressive Cyclosporin A (CyA) was studied using liposomal phospholipid membranes, freshly isolated rat hepatocytes and bile canalicular plasma membrane vesicles from rat liver. The Na⁺-dependent, saturable uptake of the bile acid ³H-taurocholate into isolated rat liver cells was apparently competitively inhibited by CyA. However, the uptake of CyA into the cells was neither saturable, nor temperature-dependent nor Na⁺-dependent, nor could it be inhibited by bile salts or CyA-derivatives, indicating passive diffusion.

In steady state depolarization fluorescence studies, CyA caused a concentration-dependent decrease of anisotropy, indicating a membrane fluidizing effect. Ion flux experiments demonstrated that CyA dramatically increases the permeability of Na⁺ and Ca²⁺ across phospholipid membranes in a dose- and time-dependent manner, suggesting a ionophoretic activity that might have a direct impact on cellular ion homeostasis and regulation of bile acid uptake.

Photoaffinity labeling with a [³H]-labeled photolabile CyA-derivative resulted in the predominant incorporation of radioactivity into a membrane polypeptide with an apparent molecular weight of 160,000 and a minor labeling of polypeptides with molecular weights of 85,000-90,000. In contrast, use of a photolabile bile acid resulted in the labeling of a membrane polypeptide with an apparent molecular weight of 110,000, representing the bile canalicular bile acid carrier. The photoaffinity labeling as well as CyA transport by canalicular membrane vesicles were inhibited by CyA and the p-glycoprotein substrates daunomycin and PSC-833, but not by taurocholate, indicating that CyA is excreted by p-glycoprotein. CyA uptake by bile canalicular membrane vesicles was ATP-dependent and could not be inhibited by taurocholate. CyA caused a decrease in the maximum amount of bile salt accumulated by the vesicles with time. However, initial rates of [³H]-taurocholate uptake within the first 2.5 min remained unchanged at increasing CyA concentrations.

In summary, the data indicate that CyA does not directly interact with the hepatic bile acid transport systems. Its cholestatic action may rather be the result of alterations in membrane fluidity, intracellular effects and an interaction with p-glycoprotein.

INTRODUCTION

The immunosuppressive peptide Cyclosporin A (CyA)^c (Sandimmun[®] [1]) is of special interest, because of its early use in the long-term treatment of autoimmune diseases and the prevention of allograft rejection after organ transplantation [1-3]. Its clinical

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^c Abbreviations: CyA, cyclosporin A; DPH, 1,6-diphenyl-1,3,5-hexatriene.

administration requires cautious monitoring due to various side effects. In addition to nephrotoxicity, increased bilirubin plasma levels and elevated serum bile salt levels have been reported from animals and patients receiving the drug [4-10], indicating cholestatic effects of CyA or its metabolites. In recent studies inhibition of bile salt biosynthesis was suggested as an explanation for the cholestatic effects seen at CyA treatment [11]. However, increased plasma levels of transaminases have not been reported [5, 6, 12, 13], indicating a transport defect rather than a general cellular impairment. An interference of CyA with the transport of bile acids is discussed controversially. With isolated rat hepatocytes nonspecific binding of CyA to protein components of a postulated cholate transport system without uptake by this system was observed [14], whereas competitive and noncompetitive inhibition of taurocholate uptake by CyA has been seen in membrane vesicle experiments [15, 16].

To shed light on these controversies, a study was performed on the hepatic uptake and excretion of CyA using different experimental models such as liposomal phospholipid membranes, freshly isolated rat hepatocytes, and bile canalicular plasma membrane vesicles from rat liver.

MATERIALS AND METHODS

Chemicals

All cyclosporins were from Sandoz Pharma AG, Basle, Switzerland. [6-³H]-taurocholate (Na⁺-salt) with a specific activity of 6.6 Ci/mmol was obtained from DuPont-New England Nuclear (Dreieich, Germany). ²²NaCl with a specific activity of 500 mCi/mg Na⁺ was purchased from Amersham (Buckinghamshire, UK). 1,6-diphenyl-1,3,5-hexatriene (DPH) was from Molecular Probes, Inc. (Eugene, OR). All other substances were obtained at the highest purity from commercial sources.

Isolation of hepatocytes

Isolated hepatocytes from livers of male Wistar rats were prepared by a modified collagenase perfusion technique [17]. The cells were suspended in Krebs-Henseleit-medium at a concentration of 2-3 x 10⁶ cells/ml. They were allowed to recover for approximately 30 min by gently shaking them under a carbogen (95 percent O₂/5 percent CO₂) atmosphere at 37°C before all uptake studies. The viability of the cells was estimated after the isolation procedure and a second time after the transport experiments by determination of Trypan blue exclusion and leakage of lactate dehydrogenase. Only cells with a viability greater than 90 percent were used for transport experiments. All experiments with freshly isolated cells were performed within 2 hr after hepatocyte isolation.

Transport experiments with isolated hepatocytes

Uptake of radiolabelled CyA and taurocholate into freshly isolated hepatocytes was measured at 37°C by a rapid centrifugation technique [18, 19]. The concentration of the CyA varied between 0.1 and 10 μM dependent upon the extreme low solubility in aqueous solutions, the bile salt concentration ranging from 0.1 to 250 μM. CyA was added to the incubation media from stock solutions of DMSO to obtain a final solvent concentration not higher than 1 percent (v/v). The solvent was also added in reference measurements without CyA. Control experiments (bile salt uptake measurements) showed no difference in uptake rates. Initial rates of uptake into isolated hepatocytes were calculated from the slopes in the linear range by linear regression analysis, considering only data points within the first 60 sec of uptake (15 sec intervals). The kinetic parameters were analyzed by the non-linear least-square regression analysis program Enzfitter 1.05 (Elsevier-Biosoft,

Cambridge, UK). The resulting kinetic data are reported as means \pm SE. Statistic differences were determined by use of the appropriate paired or unpaired t-test.

Isolation of bile canalicular plasma membrane vesicles

Canalicular liver plasma membrane subfractions were isolated in high yield by a combination of rate zonal and discontinuous sucrose density gradient centrifugation as described in detail [20, 21]. Membrane vesicles were resuspended in the appropriate buffer media (see corresponding figure legends), and aliquots were stored frozen in liquid nitrogen until use.

Transport measurements with bile canalicular plasma membrane vesicles

Transport determinations in the presence and absence of ATP were determined by the rapid filtration assay following generally the recently described experimental protocols [22]. The exact initial compositions of the intravesicular buffers as well as of the extraventricular media are described in the respective figure legends. At the indicated time points, the uptake of radioactive substrate was stopped by addition of twice 2 ml ice-cold stop solution (50 mM sucrose, 100 mM KCl, 10 mM MgSO₄, 10 mM Tris/HCl, pH 7.4). After an additional washing step, the vesicle-associated radioactivity on the filters was determined by liquid scintillation counting as described [22].

Photoaffinity labeling of bile canalicular membranes

Membrane vesicles were resuspended in 50 mM sodium phosphate buffer, pH 7.4. Aliquots of 250 μ l (~0.3- 0.5 mg protein) were incubated with the sodium salt of 7,7 azo-3 α ,12 α -dihydroxy-5 β -[3 β -³H]cholan-24-oyl)-2-aminoethanesulfonic acid or the photolabile CyA derivative (1 μ M) for 3 min in the dark at 25°C. Photolysis was carried out at 300 or 350 nm for 5 min in a rotating cylindrical cuvette made of quartz glass in a Rayonet RPM 100 photoreactor (New England Ultraviolet, Hamden, Connecticut). The unbound radioactive derivatives were removed by centrifugation of the membrane suspension at 50,000 x g for 30 min. The membrane proteins were separated by SDS-polyacrylamide gel electrophoresis and the radioactivity incorporated into the various protein bands was determined as previously described [20].

Liposome preparation

Liposomes for binding and fluorescence experiments were prepared by the extrusion technique, as described previously [23]. Dioleoyl-phosphatidylcholine was used as phospholipid. The distribution of liposome size was determined by laser light scattering at 90° with a ZetaSizer III (Malvern Instruments, London, UK).

Partition coefficient measurements

Appropriate amounts of CyA were dried from stock solutions in chloroform into Eppendorf tubes. One milliliter of liposome suspension (total lipid = 10 mg) was added to the vial and shaken for 2 hours. Concentrations of CyA in the liposomal solution were between 12 and 320 μ M. Centrifugation tubes were equilibrated over night with a corresponding aqueous concentration to avoid excessive adsorption of CyA to the tube. The suspension was centrifuged in a Beckman Ultracentrifuge TL-100 at 198,000 rpm for 6 hr at 25°C. The lipid content was measured by a phospholipase-D/cholinoxidase/PAP-test (WAKO Chemicals GmbH, Neuss, Germany). CyA concentration in supernatant and pellet was measured by a radioimmunoassay (detection limit: 15 ng/ml).

Fluorescence measurements

Steady state fluorescence measurement were performed with a Perkin-Elmer LS50B instrument (Perkin Elmer Inc. Basle, Switzerland) equipped with polarization and Biokin

accessories. The cuvette holder was controlled by a Julabo F10 thermostat (Merck ABS, Switzerland) at 25°C. Excitation was done at 336 nm (3 nm slit width), emission was determined over a 10-second integration at 430 nm (3 nm slit width). DPH was added to the liposomal suspension to give a molar lipid:DPH ratio of 200 in a buffer system of 50 mM NaCl, 50 mM KCl, 20 mM Tris, pH 7.4. The LS-50B software allowed the calculation of anisotropy with G-factor correction.

Ion flux measurements

Liposomes were suspended in 60 µl buffer containing 150 mM NaCl, 30 mM imidazole, 5 mM MgSO₄, 1 mM EDTA, pH 7.2 and 2 µCi ²²NaCl. The suspensions were pre-equilibrated with CyA. For that reason, 1 to 4 µl of the test compound stock solution (10 mM in ethanol:H₂O) was added to 60 µl liposomes in different test vials. As control experiment, the same amount of ethanol:H₂O without test compound was added to 60 µl of the liposome suspension. The flux experiments were performed at 20-23°C.

An ion exchange resin (Bio-Rex 70) was equilibrated for at least one day with 1 mM Tris, pH 7.4. For at least two days the resin was re-equilibrated with 0.15 M Tris, pH 7.4. After filling the microcolumns with the resin, the columns were washed with 2 ml of the appropriate iso-osmolar sucrose solution, containing 342 mM sucrose, 20 mM Tris, pH 7.2 for Na⁺-flux measurement. The prepared microcolumns could be stored at 4°C for several days.

The liposomes were added to the radioactive test compound, thoroughly mixed and kept at room temperature or at 37°C. At different times (0 hr to 150 hr) 6 µl of the liposome suspension was added to 60 µl of cold (0-2°C) iso-osmolar sucrose and mixed well. From that mixture, 28 µl aliquots were layered on the cooled microcolumns. The liposomes from the sample were directly eluted with 1 ml cold (0-4°C) iso-osmolar sucrose into a scintillation counting vial. Four ml of scintillation fluid were added and the radioactivity was measured for 60 min or until the relative standard deviation was less than 0.4 percent. As control experiment, 5 ml of the liposome-sucrose mixture was mixed with 1 ml of sucrose and the radioactivity was determined.

RESULTS

Cellular uptake studies

The saturable uptake of taurocholate into freshly isolated hepatocytes was decreased in the presence of increasing concentrations of CyA in the incubation medium. The type of inhibition was apparently competitive (Figure 1). The transport of CyA isolated rat hepatocytes was determined in the concentration range from 0.1 µM to 10 µM. Higher concentrations resulted in visible precipitation of the cyclopeptide due to its highly lipophilic character. After a rapid concentration dependent absorption within the first 2 to 3 sec the uptake was linear over the whole time interval of investigation (up to 5 min). When the initial rates of uptake, derived from linear regression of the values for cell associated radioactivity between 15 sec and 60 sec, were plotted as a function of increasing concentrations, no marked deviation from linearity could be observed, indicating that the uptake occurs by passive diffusion (Figure 2). The diffusion coefficient of CyA was $0.50 \pm 0.02 \times 10^{-4}$ l/min/mg protein. The uptake was neither Na⁺-dependent as was taurocholate uptake, nor temperature dependent. The transport of CyA could not be inhibited by taurocholate at concentrations up to 100 µM (Figure 2).

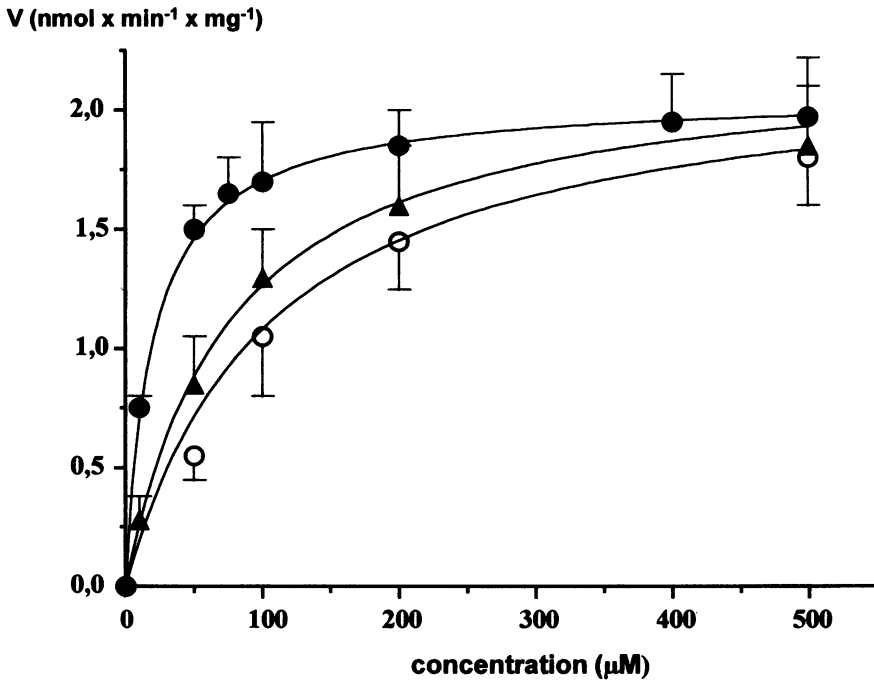


Figure 1. Initial rates of uptake of [³H]-taurocholate into freshly isolated rat hepatocytes in the absence (●) and in the presence of 0.5 µM CyA (▲) or 5.0 µM CyA (○) (n = 6; means ± S.E.).

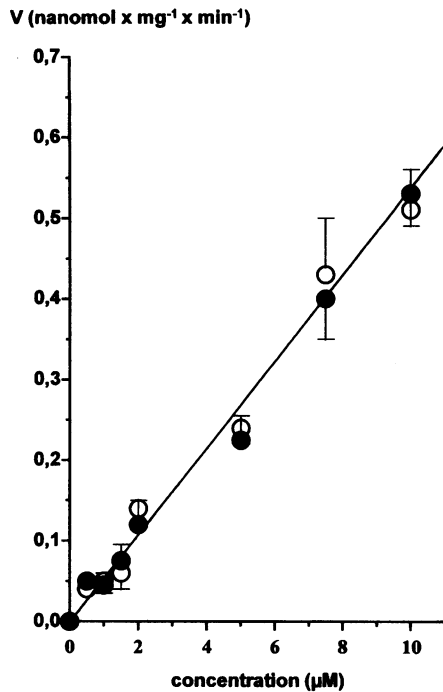


Figure 2. Initial rates of uptake of [³H]-CyA into freshly isolated rat hepatocytes in the absence (○) or in the presence (●) of 100 µM cholytaurine (n = 6; means ± S.E.).

Cyclosporin-induced changes in physicochemical membrane properties

The lipophilicity of CyA was determined by measuring the partition coefficient between liposomal membranes and buffer. The partition coefficient for CyA was 4034 ± 521 (mean \pm SD, $n = 20$). In addition, liposomal membrane anisotropy was determined. In steady-state depolarization fluorescence studies, Dioleoyl-phosphatidylcholine liposomes were incubated with increasing amounts of CyA in the concentration range between 0.5-10 μM . CyA caused a pronounced concentration-dependent decrease of anisotropy (Figure 3), indicating a membrane-fluidizing effect (decrease of 46 percent measured at 25°C at a lipid:drug ratio of 13:1). Furthermore, Na^+ and Ca^{2+} -fluxes across liposomal membrane were determined. The permeability of liposomal membranes for Na^+ and Ca^{2+} ions was dramatically increased in a dose- and time-dependent manner in the presence of CyA (Figure 4), suggesting an ionophoric activity of the latter.

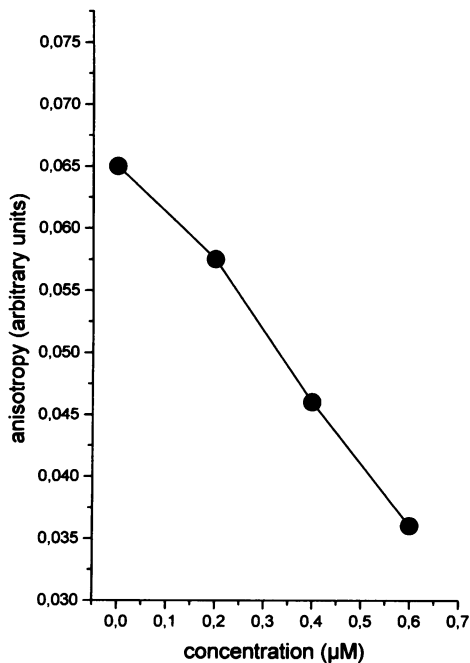


Figure 3. Steady state fluorescence anisotropy of DOPC liposomes incubated with different amounts of cyclosporins. Liposomes (2 mg phospholipid/3 ml buffer: 50 mM NaCl, 50 mM KCl, 20 mM Tris; pH 7.4) were preincubated with DPH (molar ratio phospholipid:DPH = 200:1) for about 1 hr. CyA was added from a 100 mM stock solution in ethanol. Measurements (excitation 336 nm, emission 430 nm, 10 sec integration, 3 nm slit width) were taken at 25°C. Ethanol solution without cyclosporins did not show any significant changes in anisotropy.

Photoaffinity labeling studies with bile canalicular membrane vesicles

Photoaffinity labeling of bile canalicular membrane vesicles from rat liver with a photolabile ^3H -labelled CyA-derivative resulted in the incorporation of radioactivity into a polypeptide with apparent molecular weight of approximately 160,000 and in a minor labeling of polypeptides in the molecular weight range of 85,000-90,000 (Figure 5a). The extent of labeling of the polypeptide with the apparent molecular weight of 160,000 was reduced when the experiment was performed in the presence of unlabeled CyA or in the presence of PSC-833, a potent inhibitor of p-glycoprotein. Taurocholate (100 μM) had no effect on the extent of the labeling (Figure 5a). In contrast, when a photolabile bile salt derivative [Na^+ -salt of (7,7 azo-3 α ,12 α -dihydroxy-5 β -[3 β - ^3H]cholan-24-oyl)-2-aminoethanesulfonic acid] was used, the canalicular bile salt carrier protein with an apparent molecular weight of 110,000 was almost exclusively labeled. The extent of this labeling was only slightly reduced by 0.5 - 5 μM CyA. A significantly lower extent of labeling was seen, when the experiment was performed in the presence of 100 μM taurocholate (Figure 5b).

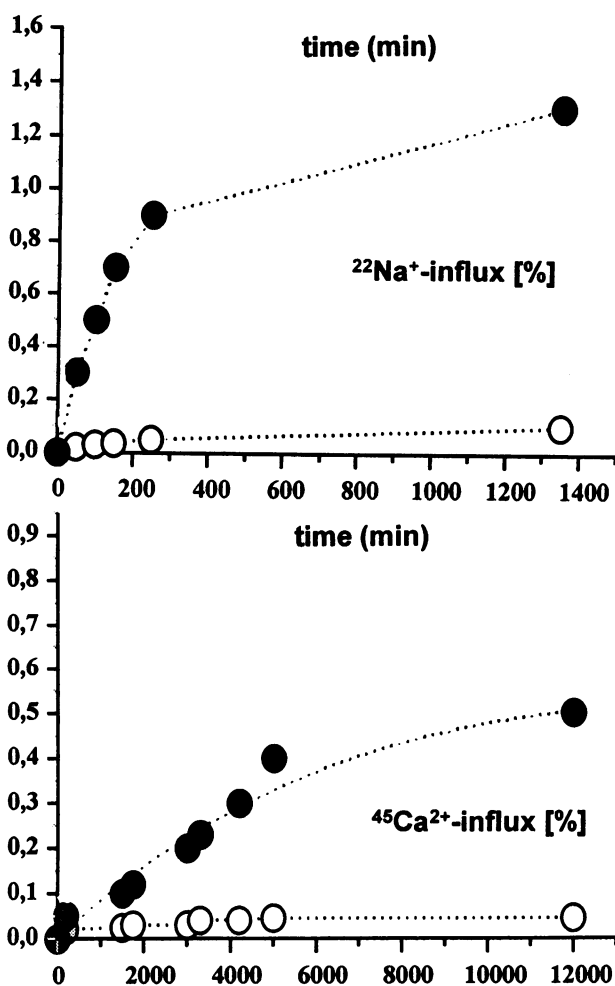


Figure 4. A (upper): Time dependent Na^+ -flux measurements with liposomes loaded with $480 \mu\text{M}$ CyA (●); ○ = blank. **B (lower):** Time dependent Ca^{2+} -flux measurements with liposomes loaded with $500 \mu\text{M}$ CyA (●); ○ = blank. The given concentrations of CyA are concentrations in the total lipid suspension; the concentrations in the aqueous solution were approximately $20 \mu\text{M}$. Error bars are covered by the symbols.

Kinetic studies were performed in order to support the photoaffinity labeling experiments and to clarify whether CyA only binds to membrane proteins or whether it is a transported substrate. When uptake of CyA was studied, the bile canalicular membrane vesicles showed an ATP-dependent transport (Figure 6), which could be inhibited by daunomycin, a p-glycoprotein substrate. Taurocholate had only minor effects upon CyA uptake rates in the vesicles. When the ATP-dependent transport of ^3H -taurocholate was determined, the presence of CyA resulted in a decrease of the maximum amount of bile salt accumulated in the vesicles with time. However, the initial rates of ^3H -taurocholate uptake within the first 2.5 min of determination remained unchanged at increasing CyA concentrations (Figure 7).

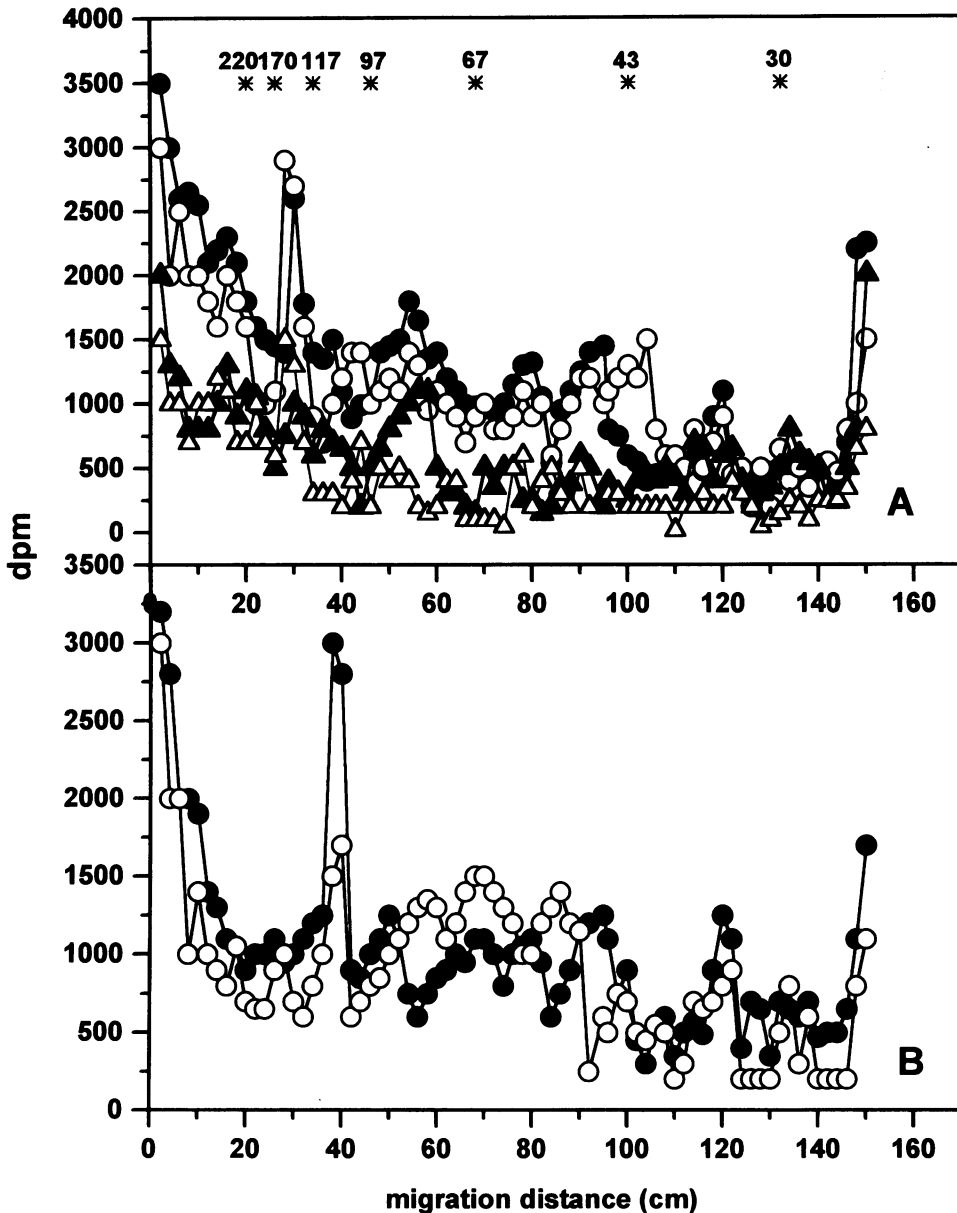


Figure 5. A (upper): Photoaffinity labeling of bile canaliculi membrane vesicles from rat liver with a photolabile [³H]-CyA-derivative in the absence of 100 μM taurocholate (●) and in the presence of 100 μM taurocholate (○), 10 μM non-immunosuppressive PSC-833 (▲) or 10 μM CyA (Δ). The photolabile compound is predominantly incorporated into a polypeptide with apparent molecular weights of 160,000. **B (lower):** Photoaffinity labeling of bile canaliculi membrane vesicles from rat liver with a photolabile [³H]-taurocholate derivative in the absence (●) or in the presence of 100 μM taurocholate (○). The bile salt is almost exclusively incorporated into a polypeptide with an apparent molecular weight of 110,000.

picomoles / mg protein

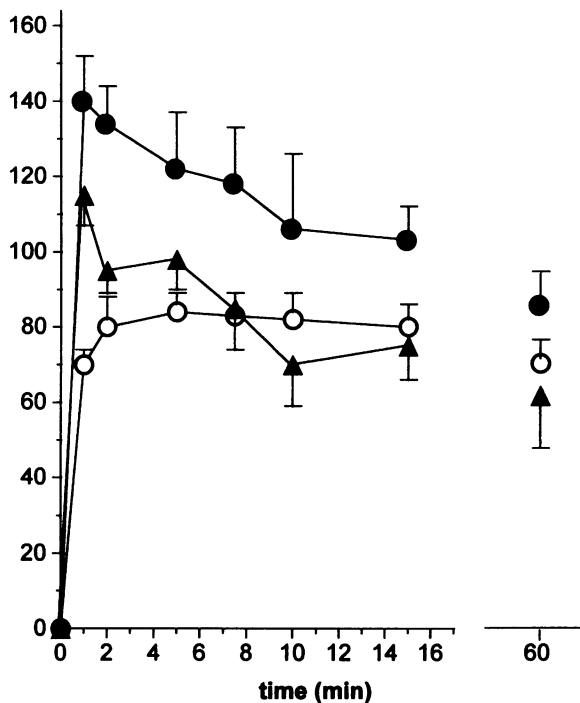


Figure 6. Uptake of [^3H]-CyA into bile canicular membrane vesicles from rat liver. The concentration of [^3H]-CyA in the incubation medium is $6.1 \mu\text{M}$. Uptake in the absence (\circ) or in the presence (\bullet) of 1 mM ATP or in the presence of 1 mM ATP and $100 \mu\text{M}$ daunomycin (\blacktriangle).

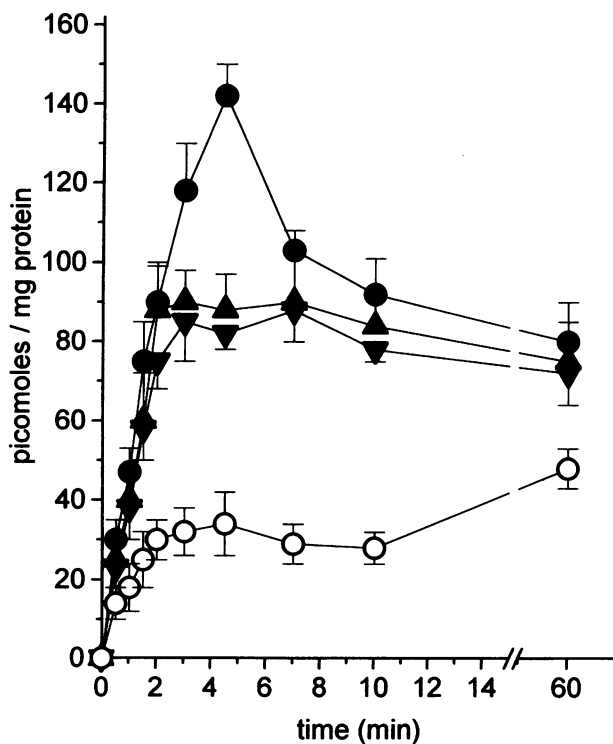


Figure 7. Uptake of [^3H]-taurocholate into bile canicular membrane vesicles from rat liver. The concentration of [^3H]-taurocholate in the incubation medium is $1 \mu\text{M}$. Uptake in the absence (\circ) or in the presence (\bullet) of 1 mM ATP or in the presence of $0.5 \mu\text{M}$ (\blacktriangle) or $5 \mu\text{M}$ (\blacktriangledown) CyA and 1 mM ATP.

DISCUSSION

Cyclosporin A, a cyclic undecapeptide produced by the fungus *Tolypocladium inflatum* Gams, is a powerful immunosuppressant drug [1], which selectively inhibits the interleukin-2 (IL-2)-driven proliferation of activated T-lymphocytes. The pharmacokinetics of CyA exhibit a high inter- and intraindividual variability, which is dependent upon various factors such as food interactions, influence of bile, and physicochemical parameters, e.g., the high lipophilicity or differences in drug metabolism caused by the patient's metabolic situation [24]. The immunosuppressive therapy may be associated with adverse effects including renal dysfunction, vascular damage and hepatic side effects [5, 25]. CyA-induced increases of serum bile acid levels in animal and man [10, 12, 26] are indicative for a certain cholestatic potential. Since the data on hepatic transport of CyA are controversial [14-16], we investigated the transport of that important drug in more detail in several *in vitro* models.

CyA has a unique lipophilicity making effects upon cellular membranes most likely, for example, the anisotropy decrease correlates with a dramatic increase of the membrane permeability of Na^+ and Ca^{2+} . Both effects, the changes in membrane fluidity as well as in ion homeostasis, may contribute to the decrease of hepatic bile acid uptake during CyA treatment by disturbing the transmembrane potential and cellular ion gradients. Similar effects have also been observed in other cellular systems [27, 28]. In human lymphocytes CyA caused an enhanced K^+ efflux and reduced the membrane potential. Therefore it is likely, that such alterations in membrane integrity also contribute to CyA-induced disturbances of liver function.

The increase of serum bile acid levels in patients observed during CyA-treatment indicates a disturbance of hepatic bile acid transport. In our study, CyA had a pronounced effect on taurocholate uptake with an apparently competitive type of inhibition. For the present, this confirms previous findings with rat hepatocyte basolateral plasma membranes and hepatocyte primary cultures, where a competitive inhibition was also observed [15, 29]. However, CyA itself showed no sign of active uptake by isolated rat hepatocytes. Therefore, we conclude that the inhibition of bile acid transport is the result of an unspecific binding, the observed disturbance in ion homeostasis, and a simple rapid partitioning into the lipid phase. The present finding is supported by findings in isolated intact kidney tubules, where a passive uptake of cyclosporins from the basolateral side and an active secretion at the luminal side was observed [30].

Previous studies indicated that CyA is a potent inhibitor of ATP-dependent transport processes at the canalicular membrane including p-glycoprotein mediated transport [31-34]. Here, we demonstrate that CyA itself can be transported across bile canalicular membranes by an active, ATP-dependent mechanism. The photoaffinity labeling experiment and the kinetic experiment give clear evidence that it is a substrate for p-glycoprotein. The labeling was decreased in the presence unlabeled CyA and of the p-glycoprotein substrate PSC-833, which is a non-immunosuppressive cyclosporin derivative with a very high affinity to p-glycoprotein [35-37]. Our findings are in accordance with kinetic and photoaffinity labeling experiments in kidney tubular cells [30, 38, 39], kinetic studies with the intestinal cell line Caco-2 [40] and at the brain capillary endothelium [41], all cell types expressing p-glycoprotein and exhibiting active CyA transport at their luminal side. The fact, that the initial rates of bile acid transport by the canalicular membrane vesicles remain unchanged over the first 2.5 min, suggests that CyA transport results rather in a faster use of the cellular ATP pool than in a direct competition of the drug at the bile acid carrier.

SUMMARY

The present studies indicate that CyA enters hepatocytes by passive diffusion and is secreted by p-glycoprotein. Inhibition of bile salt transport does not occur due to a specific interaction. For an estimation of the clinical relevance of these findings, the pharmacokinetic properties of CyA should be verified in primate or human studies with emphasis on the p-glycoprotein interaction of the drug.

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