Biochemical Pharmacology, Vol. 42, No. 1, pp. 193–195, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

Substrate transport and cyclosporin A in isolated rat hepatocytes

(*Received* 26 *November* 1990; *accepted* 18 *February* 1991)

The cholestatic effect of cyclosporin A (CsA*) has been observed in experimental animals as well as in man [1]. CsA induces depressed bile acid transport *in vitro* [2–7] and elevations of serum bile salts and bilirubin together with reduced bile flow *in vivo* [8–12]. These effects are apparent without appreciable signs of cell damage. Furthermore, CsA inhibits protein synthesis [13] and mitochondrial respiration [14] in cultured hepatocytes. However, it is still unclear whether these effects are associated with the mechanism of CsA-induced reduction of bile flow. Inhibition of bile acid transport may be of importance [1, 15] since the precise mechanism is still unknown.

Direct interaction between CsA and bile salt transport protein has been suggested [4, 16], because inhibition of bile acid uptake was apparent immediately after exposure of hepatocytes to CsA. However, longer exposure to CsA induces alterations in rat hepatocyte basolateral membrane lipid composition and results in decreases of membrane fluidity as well as taurocholate uptake [17]. Moreover, the CsA molecule is so lipophilic that it is incorporated almost instantaneously into membrane lipid [16] and its effect has been shown to be difficult to remove [7] although it can be reversed under appropriate conditions [4]. Therefore, it is probable that CsA may partition into and induce non-specific perturbation in membrane lipid and consequently diminish transport protein activity. Nonetheless, as CsA did not depress uptake of α-aminoisobutyric acid [2, 3], any generalized effect of CsA on plasma membrane requires further appraisal. For this reason, the effects of CsA on the hepatocellular transport of another substrate (galactose) was evaluated, along with effects on counter transport of taurocholate and activation energy for transport of substrates.

Materials and Methods

Tauro[*carbonyl*-^{14}C]cholic acid, sodium salt, [^{14}C]-galactose and [21,22-^{3}H]ouabain were purchased from Amersham (Sydney, NSW). Unlabelled substrates and collagenase (type IV) were from Sigma (Sigma Chemical Co., St Louis, MO, U.S.A.). Cyclosporin A was a gift from Sandoz Australia Pty Ltd (Sydney, NSW).

Male Sprague–Dawley rats weighing about 300–400 g (10–14 weeks of age) from The University of Sydney Animal House were given free access to food and water. Isolated hepatocytes were prepared by the method of Berry and Friend [18] with modifications including a Percoll centrifugation step [4]. Viability of hepatocytes was determined by trypan blue exclusion, which was between 94 and 98%. Cells were suspended in Hank's buffer supplemented with 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hank-HEPES) as the incubation buffer at cell densities of 1.4×10^6 and 10×10^6 cells/mL for uptake and efflux experiments, respectively. Cells stored on ice for up to 3 hr were re-equilibrated at 37° or other designated temperatures for 15 min before use. Transport experiments for galactose were performed at 27° throughout as the slower rate at the lower temperature is easier to measure.

Uptake and efflux experiments were performed essentially according to our previous reports [3, 19]. Briefly, for the uptake experiments, cells were incubated at predetermined temperatures for 15 min before an addition of radiolabelled substrate. Samples of cell suspensions were taken at appropriate times and cells and medium were separated by a rapid centrifugation technique [20]. For efflux experiments, cells were preloaded with galactose for 5 min before being diluted into a substrate-free medium and cell suspensions were sampled as described above.

Initial rates of uptake were calculated over the period where uptake was linear with time (15–45 sec for taurocholate, 1–5 min for ouabain and 15–30 sec for galactose). Activation energies for transport of taurocholate and ouabain were calculated from the Arrhenius plot of the natural log of initial velocity of substrate uptake (10 μM taurocholate and 125 μM ouabain) versus 1/temperature in degrees Kelvin (between 26–37°C). Values are the product of the slope of the plot and the gas constant.

In counter transport experiments, hepatocyte suspensions of 1.4×10^6 cells/mL were preloaded at 37° with [^{14}C]taurocholate for 15 min before addition of unlabelled taurocholate, CsA or taurocholate plus CsA to the final concentration of 100 μM for each agent. Normal saline (100 μL) and dimethylsulfoxide (10 μL) were vehicle controls for unlabelled taurocholate and CsA, respectively. Samples were taken, cells and medium separated and then assayed for radioactivity.

The data were analysed for statistical significance by an analysis of variance with *post hoc* Duncan's test. The level of significance chosen was $P < 0.05$.

Results

Uptake of galactose is extremely rapid and not significantly changed by the presence of CsA (Table 1). Efflux from the preloaded cells was also unaffected by CsA at any time up to 60 sec (Table 1). Counter transport in cells preloaded with [^{14}C]taurocholate was observed in the presence of unlabelled taurocholate, as expected. CsA did

Table 1. Effect of CsA on uptake and efflux of galactose

	CsA (μM)			
	Control	1	10	100
Uptake (nmol/mg protein)				
15 sec	100 ± 8	107 ± 2	115 ± 7	124 ± 8
30 sec	123 ± 6	125 ± 5	132 ± 3	139 ± 14
45 sec	131 ± 13	131 ± 10	141 ± 4	157 ± 19
Efflux (nmol/mg protein)				
15 sec	18.3 ± 2.9	20.7 ± 3.8	19.3 ± 2.9	20.0 ± 3.8
30 sec	14.0 ± 2.6	16.0 ± 3.0	16.0 ± 2.6	17.0 ± 3.2
45 sec	13.7 ± 2.0	14.3 ± 2.7	15.0 ± 2.3	15.0 ± 2.3
60 sec	11.0 ± 1.5	12.0 ± 1.5	12.0 ± 2.1	13.3 ± 1.8

Each value is mean ± SE of three experiments.

* Abbreviation: CsA, cyclosporin A.

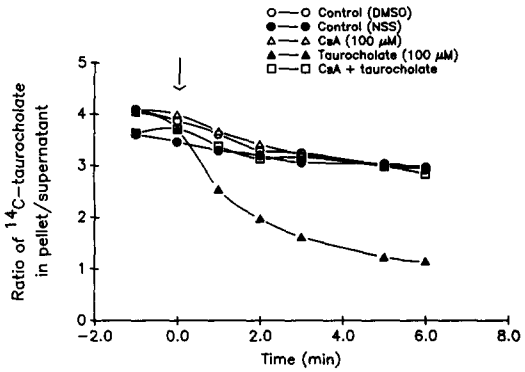

Fig. 1. Counter transport of taurocholate. Freshly isolated hepatocytes were incubated with 10 μM [^{14}C]taurocholate for 15 min before addition of unlabelled substrates or normal saline (NSS) or dimethyl sulphoxide (DMSO) as vehicle control (indicated by an arrow).

not produce counter transport of taurocholate but did inhibit the response invoked by unlabelled bile acid (Fig. 1). Activation energy for transport of [^{14}C]taurocholate in the presence of CsA or unlabelled taurocholate was unchanged (Fig. 2a), whilst that of ouabain was decreased in response to CsA (Fig. 2b).

Discussion

Uptake of galactose by isolated hepatocytes is characterized by carrier-mediated transport [21] and is unaffected by CsA. On the other hand, CsA has been reported to inhibit uptake of other carrier mediated transport systems including cholic acid [7], taurocholate [2–4] and ouabain [3]. Thus, a lack of effect on carrier transport systems for galactose and some other substrates (2-aminoisobutyric acid and $CdCl_2$ [2, 3]) may imply that the mechanism of the inhibitory effect on transport is selective to certain transport systems rather than a nonspecific action on membrane carrier proteins in general.

Counter transport is one of the characteristics of a carrier-mediated process [22] and transport of bile acids by hepatocytes has been shown previously to occur by this mechanism [23]. The lack of an effect of CsA on counter transport of taurocholate is evidence that it does not share the carrier-mediated transport system of this bile acid. However, this interpretation requires caution as CsA is known to inhibit taurocholate efflux [3].

Activation energy of transport which reflects temperature dependence of the mobility of the carrier [24] was unchanged for taurocholate but decreased for ouabain in the presence of CsA (Fig. 2a and b). The unchanged activation energy with taurocholate as substrate indicates that CsA does not cause a change in membrane fluidity, while the data with ouabain as substrate are consistent with increased membrane fluidity [25]. This might suggest that

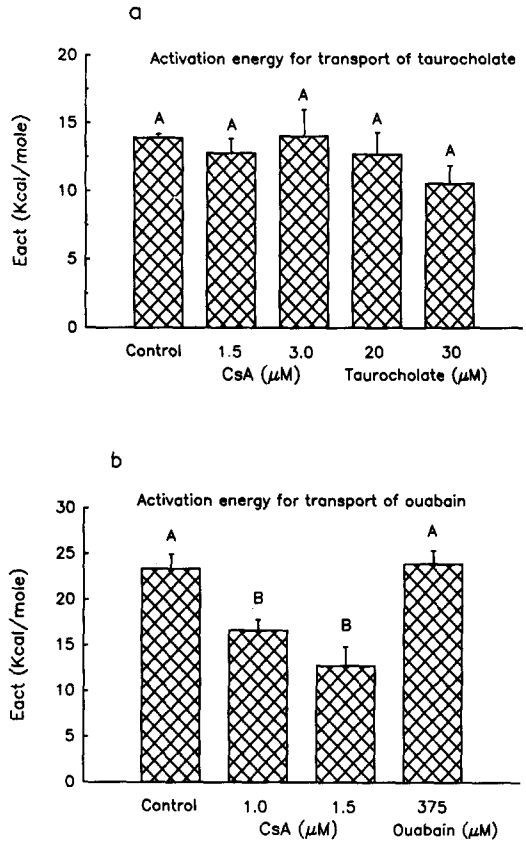

Fig. 2. Effects of CsA and unlabelled substrate on activation energy for transport of: (a) taurocholate (10 μM); and (b) ouabain (125 μM). Initial rate of uptake was determined at incubation temperature of 26–37°. Values are means ± SE (N = 3–7). Values with the same capital letter are not significantly different.

CsA causes changes in fluidity in certain microdomains of membranes. Alternatively, it may reflect the nature of the inhibition as CsA inhibits uptake of taurocholate competitively [3–5] while it inhibits transport of ouabain in a noncompetitive manner [3].

In summary, CsA has been demonstrated to have no effect on hepatocellular transport of galactose, while it is known to interfere with that of other substrates such as taurocholate. However, no effect of CsA on counter transport of taurocholate was observed, which suggests that this drug and bile salt do not share the same carrier. CsA does not affect activation energy for the transport of taurocholate while that for ouabain is decreased. Together the data indicate that CsA acts by a mechanism involving particular components of hepatocyte transport rather than by a general perturbation of cell membranes.

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REFERENCES

1. Mason J, Pharmacology of cyclosporine (Sandimmune). VII. Pathophysiology and toxicology of cyclosporine in humans and animals. *Pharmacol Rev* **42**: 423–434, 1989.
2. Boelsterli UA, Bouis P, Brouillard J-F and Donatsch P, *In vitro* toxicity assessment of cyclosporin A and its analogs in a primary rat hepatocyte culture model. *Toxicol Appl Pharmacol* **96**: 212–221, 1988.
3. Stacey NH and Kotecka B, Inhibition of taurocholate and ouabain transport in isolated rat hepatocytes by cyclosporin A. *Gastroenterology* **95**: 780–786, 1988.
4. Kukongviriyapan V and Stacey NH, Inhibition of taurocholate transport by cyclosporin A in cultured rat hepatocytes. *J Pharmacol Exp Ther* **247**: 685–689, 1988.
5. Moseley RH, Johnson TR and Morrissette JM, Inhibition of bile acid transport by cyclosporine A in rat liver plasma membrane vesicles. *J Pharmacol Exp Ther* **253**: 974–980, 1990.
6. Zimmerli B, Valantinas J and Meier PJ, Multispecificity of Na^+-dependent taurocholate uptake in basolateral (sinusoidal) rat liver plasma membrane vesicles. *J Pharmacol Exp Ther* **250**: 301–308, 1989.
7. Ziegler K and Frimmer M, Cyclosporin A and a diaziridine derivative inhibit the hepatocellular uptake of cholate, phalloidin and rifampicin. *Biochim Biophys Acta* **855**: 136–142, 1986.
8. Atkinson K, Biggs J, Dodds A and Concannon A, Cyclosporine-associated hepatotoxicity after allogenec marrow transplantation in man: differentiation from other causes of posttransplant liver disease. *Transplant Proc* **15**: 2761–2767, 1983.
9. Farthing MJG, Clark ML, Pendry A, Sloane J and Alexander P, Nature of the toxicity of cyclosporin A in the rat. *Biochem Pharmacol* **30**: 3311–3316, 1981.
10. Laupacis A, Keown PA, Ulan RA, Sinclair NR and Stiller CR, Hyperbilirubinaemia and cyclosporin A levels. *Lancet* **2**: 1426–1427, 1981.
11. Schade RR, Guglielmi A, Van Thiel DH, Thompson ME, Warty V, Griffitin B, Sanghvi A, Bahnson H and Hardesty R, Cholestasis in heart transplant recipients treated with cyclosporin. *Transplant Proc* **15**: 2757–2760, 1983.
12. Stone BG, Udani M, Sanghvi A, Warty V, Plocki K, Bedetti CD and Van Thiel DH, Cyclosporin A-induced cholestasis. The mechanism in a rat model. *Gastroenterology* **93**: 344–351, 1987.
13. Bouis P, Brouillard J-F, Fischer V, Donatsch P and Boelsterli UA, Effect of enzyme induction on Sandimmun® (cyclosporin A) biotransformation and hepatotoxicity in cultured rat hepatocytes and *in vivo*. *Biochem Pharmacol* **39**: 257–266, 1990.
14. Fournier N, Ducet G and Crevat A, Action of cyclosporine on mitochondrial calcium fluxes. *J Bioenerg Biomembr* **19**: 297–303, 1987.
15. Kukongviriyapan V and Stacey NH, Chemical-induced interference with hepatocellular transport. Role in cholestasis. *Chem Biol Interact* **77**: 225–261.
16. Ziegler K, Polzin G and Frimmer M, Hepatocellular uptake of cyclosporin A by simple diffusion. *Biochim Biophys Acta* **938**: 44–50, 1988.
17. Whitington PF, Dudeja P, Hecht JR, Whitington SH and Brasitus TA, Lipid alterations in the hepatocyte basolateral sinusoidal membrane (BLM) domain may explain cyclosporine (CsA) induced cholestasis in the rat. *Hepatology* **8**: 1363, 1988.
18. Berry MN and Friend DS, High yield preparation of isolated rat liver parenchymal cells; a biochemical and fine structural study. *J Cell Biol* **43**: 506–520, 1969.
19. Kukongviriyapan V and Stacey NH, Comparison of uptake kinetics in freshly isolated suspensions and short-term primary cultures of rat hepatocytes. *J Cell Physiol* **140**: 491–497, 1989.
20. Eaton DL and Klaassen CD, Carrier-mediated transport of ouabain in isolated hepatocytes. *J Pharmacol Exp Ther* **205**: 480–488, 1978.
21. Baur H and Heldt HW, Transport of hexose across the liver-cell membrane. *Eur J Biochem* **74**: 397–403, 1977.
22. Schafer JA and Barfuss DW, Mechanisms of transmembrane transport in isolated cells and their experimental study. *Pharmacol Ther* **10**: 223–260, 1980.
23. Anwer MS, Kroker R and Hegner D, Cholic acid uptake into isolated rat hepatocytes. *Hoppe Seylers Z Physiol Chem* **357**: 1477–1486, 1976.
24. Kletzien RF and Perdue JF, Sugar transport in chick embryo fibroblasts. I. A functional change in the plasma membrane associated with the rate of cell growth. *J Biol Chem* **249**: 3366–3374, 1974.
25. Berr F, Simon FR and Reichen J, Ethynylestradiol impairs bile salt uptake and Na-K pump function of rat hepatocytes. *Am J Physiol* **247**: G437–G443, 1984.

Biochemical Pharmacology, Vol. 42, No. 1, pp. 195–198, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

Murine strain differences in pulmonary bleomycin metabolism

(*Received* 15 *September* 1990; *accepted* 8 *February* 1991)

Bleomycin (BLM*) is an important antitumor agent that also produces scleroderma-like skin changes and potentially fatal pulmonary fibrosis [1]. The mechanism(s) of these side-effects has not been clearly established, but may be caused by BLM-mediated radical abstraction of DNA or membranes sites. Protection from toxicity may be accorded by intracellular metabolism of BLM to the less active deamidoBLM (dBLM) form by the enzyme BLM hydrolase [2–4]. The metabolite produced by BLM hydrolase is approximately 1% as efficient as the parent compound in producing oxygen radicals, DNA strand scissions, tumor cytotoxicity, or pulmonary fibrosis in animal models [4].

* Abbreviations: BLM, bleomycin; BLM A_2, bleomycin A_2; dBLM, deamidobleomycin; dBLM A_2, deamidobleomycin A_2; PBS, phosphate-buffered saline; and i.t., intratracheal.