Biochimica et Biophysica Acta, 778 (1984) 539–548
Elsevier

BBA 72364

DRIVING FORCES IN HEPATOCELLULAR UPTAKE OF PHALLOIDIN AND CHOLATE

ERNST PETZINGER * and MAX FRIMMER

Institute of Pharmacology and Toxicology, Justus Liebig University Giessen, Frankfurter Strasse, 107, D-6300 Giessen (F.R.G.)

(Received April 16th, 1984)

Key words: Membrane potential; Phalloidin transport; Cholate transport; Bile acid; Na^+ dependence; (Rat liver)

Active uptake of phalloidin and cholate in isolated rat liver cells depends upon both Na^+ gradient and membrane potential. Omission of Na^+ or inhibition of the ($Na^+ + K^+$)-ATPase diminished both phalloidin and cholate uptake. Dissipation of the sodium, potassium or proton gradient by monensin, nigericin, gramicidin and valinomycin blocked phalloidin uptake and also caused reduction of cholate transport. Chelation of Ca^{2+} and Mg^{2+} by EGTA or incubation of liver cells with NH_4Cl neither influenced phalloidin nor cholate uptake. Hyperpolarization of liver cells by the lipophilic anions NO_3^- or SCN^- enhanced phalloidin but reduced cholate uptake. Depolarization induced by a reversed K^+ gradient reduced both kinds of transport. The results indicate that sodium ions and the membrane potential are driving forces for phalloidin and cholate uptake in hepatocytes.

Introduction

The uptake of bile acids in hepatocytes occurs by both sodium -dependent and -independent pathways [1–5]. The sodium-dependent transport is thought to be mediated by a specific carrier which enables hepatocytes to concentrate bile acids. Accumulative bile acid uptake exhibits all characteristics of a secondary active carrier mediated transport.

The sodium-independent system was proved to be less substrate specific because of its alternative availability for bilirubin, bromosulfophthalein and indocyanin green [6–9]. Several lines of evidence suggest that the cyclopeptide phalloidin is also taken up by one of these transport systems [10]. The present study was conducted to find out if the phalloidin uptake is a secondary active transport similar to the sodium-dependent uptake of bile acids. Emphasis was placed upon the energenization of the apparently active phalloidin uptake. For comparison the uptake of cholate was studied under identical conditions.

Some results have been presented previously at the spring meeting of the German Pharmacological Society 1981 [38]. The present paper was part of the Habilitationsschrift of E.P. in 1983.

Materials and Methods

Preparation of hepatocytes. Liver cells were isolated according to Ref. 11 by perfusion of rat liver with 0.05% collagenase (Boehringer Mannheim) in Krebs-Henseleit buffer in the absence of Ca^{2+}. Details of the isolation procedure have been previously described [12,13]. Isolated hepatocytes were equilibrated for 30 min in Tyrode buffer, pH 7.4 at 37°C in O_2/CO_2 (95 : 5) atmosphere and used within 2 h. 1 ml cell suspension contained $2 \cdot 10^6$ hepatocytes corresponding to 3.8 mg cell protein. Most of the isolated cells (85–90%) were viable as judged by 0.2% trypan blue. Protein determination

* To whom reprint requests should be addressed.

was performed according to the biuret method with bovine serum albumin as a standard.

Measurement of uptake. Phalloidin uptake was measured by adding a mixture of demethyl[^{3}H]phalloin and phalloidin, final concentration 50–100 nM/6 μM to 1 ml liver cell suspension according to Ref. 14. Demethyl[^{3}H]phalloin (spec. act. 129 GBq/mmol) was from Professor Dr. Th. Wieland's laboratory, [^{14}C]cholate (spec. act. 1.92 GBq/mmol, Amersham-Buchler) was added to 1 ml cell suspension at a final concentration of 1 μM. At the indicated time aliquots of 100 μl cell suspension were withdrawn and centrifuged through silicon oil according to Ref. 15. This technique was used throughout the uptake experiments to stop the reaction within 1–2 s. The radioactivity of the sedimented cells was measured in Lipoluma/Lumasolve/Water mixture (100 : 10 : 2, v/v, Baker Chemicals, Phillipsburg, NJ, U.S.A.).

Experiments with ionophores. Ionophores were dissolved in ethanol to yield a stock solution of 1 mg/ml. In the cell suspension the ethanol concentration was between 1 and 2% and had no measurable effect on phalloidin or cholate uptake.

Experiments in substituted buffer media. Isolated hepatocytes were stored at 37°C under O_2/CO_2 atmosphere in Tyrode buffer. When other buffers were used hepatocytes were carefully centrifuged in an Eppendorf table centrifuge (centrifugation time: 1 s) and resuspended in the appropriate buffer. The following buffers were used:

Tyrode-buffer (NaCl): 137 mM NaCl, 2.7 mM KCl, 1.05 mM $MgCl_2$, 1.8 mM $CaCl_2$, 12 mM $NaHCO_3$, 5.55 mM glucose, 0.42 mM KH_2PO_4; pH adjusted with 1 M HCl to 7.4.

K^+-buffer: 137 mM KCl, 2.7 mM KCl, 1.05 mM $MgCl_2$, 1.8 mM $CaCl_2$, 12 mM $LiHCO_3$, 5.55 mM glucose, 0.42 mM KH_2PO_4; pH adjusted with 1 M HCl to 7.4.

Li^+-buffer: 137 mM LiCl, 2.7 mM KCl, 1.05 mM $MgCl_2$, 1.8 mM $CaCl_2$, 12 mM $LiHCO_3$, 5.55 mM glucose, 0.42 mM KH_2PO_4; pH adjusted with 1 M HCl to 7.4.

$Choline^+$-buffer: 137 mM choline chloride, 2.7 mM KCl, 1.05 mM $MgCl_2$, 1.8 mM $CaCl_2$, 12 mM $LiHCO_3$, 5.55 mM glucose, 0.42 mM KH_2PO_4; pH adjusted with concentrated HCl to 7.4.

SCN^-, NO_3^-, SO_4^{2-}-buffer: 137 mM NaCl was substituted in Tyrode buffer by 137 mM NaSCN, $NaNO_3$ or Na_2SO_4, respectively. The pH was adjusted with 0.5 M H_2SO_4 or 1 M HNO_3 to pH 7.4. Membrane depolarization by KCl-Tyrode was achieved by substitution of NaCl by equimolar concentration of KCl. $NaHCO_3$ was not substituted in these buffers in constrast to the above described experiments.

K^+ analysis. K^+ was measured in an Eppendorf flame photometer in the supernatant of hepatocytes after centrifugation at 50 $\times g$. 10% trichloracetic acid was added to the supernatant prior to flame photometry.

Determination of the ATP content of hepatocytes. The ATP content of isolated hepatocytes was measured by an enzymatic method with hexokinase/glucose-6-phosphate dehydrogenase [16]. 1 ml cells ($20 \cdot 10^6$ hepatocytes/ml) were washed three times in Tyrode buffer to remove ATP released by damaged cells and boiled for 2 min in 9 mM perchloric acid. The samples were stored in liquid nitrogen. ATP was measured at 366 nm wavelength in the supernatant of the samples after centrifugation of 50 $\times g$. The cuvette contained: 250 μl triethanolamine buffer (50 mM, pH 7.5), 13 μl NADP (10 mM in H_2O bidest.), 26 μl $MgCl_2$ (100 mM in H_2O bidest.), 100 μl sample, 5 μl glycose-6-phosphatedehydrogenase (1 mg/ml diluted in 2.125 ml 3 M ammonium sulfate solution), 40 μl glucose (500 mM) and 5 μl hexokinase (2 mg/ml diluted in 2.125 ml 3 M ammonium sulfate solution). All enzymes were obtained from Boehringer Mannheim.

Materials. Monensin was a gift of Elli Lilly Co., Bad-Homburg. Nigericin, furosemide and valinomycin were from Sigma Chemical Co. St. Louis. Ouabain was purchased from SERVA, Heidelberg, phalloidin was from Dr. Madaus and Co., Cologne. Enzymes and substrates for ATP determination were from Boehringer, Mannheim, all other chemicals from Merck, Darmstadt. Buffer salts were of p.A. grade. Gramicidin derivatives 1–7 were kindly supplied by Professor Dr. E. Gross, Bethesda, MD, U.S.A.

Gramicidin 4; Valine-gramicidin A

HCO-NH-(L-Val^1)-(Gly^2)-(L-Ala^3)-(D-Leu^4)-(L-Ala^5)-(D-Val^6)-(L-Val^7)-(D-Val^8)-(L-Try^9)-(D-Leu^{10})-(L-Try^{11})-(D-Leu^{12})-(L-Try^{13})-(D-Leu^{14})-(L-Try^{15})-NH-CH_2-CH_2OH.

Gramicidin 1: Each D-amino acid of gramicidin A is substituted by glycin
HCO-NH-(L-Val^1)-(Gly^2)-(L-Ala^3)-(Gly^4)-(L-Ala^5)-(Gly^6)-(L-Val^7)-(Gly^8)-(L-Try^9)-(Gly^{10})-(L-Try^{11})-(Gly^{12})-(L-Try^{13})-(Gly^{14})-(L-Try^{15})-NH-CH_2-CH_2OH.
Gramicidin 2: Like 1 but with BOC-group instead of the formyl-group
Gramicidin 5: Methionine-gramicidin A. Like 4, but with L-methionine instead of L-valine at the first amino acid position.
Gramicidin 6: HCO-NH-((L-Trp)-(D-Leu))$_7$-(L-Trp)-NH-CH_2-CH_2OH
Gramicidin 7: Gramicidin C, contains tyrosin instead of tryptophan at position 11.
Gramicidin D: Commercially available mixture of natural gramicidin A, B and C with unknown mixing ratios.

Statistics. Experimental values are given by $\bar{x} \pm$ S.D. Statistic means were compared with Student's two-tailed t-test, $P \leq 0.05$ is taken as significantly different result. P values were calculated from the equation of the t-distribution.

Results

Effect of Na^+, Ca^{2+} and Mg^{2+} on the uptake of phalloidin and cholate into hepatocytes

In most secondary active transport systems of eucaryotes sodium is the major cation which serves for the uphill transport of organic solutes [17]. When external Na^+ was substituted by Li^+, K^+ or choline$^+$ the uptake of phalloidin and cholate was reduced (Fig. 1). This inhibition was reversed if liver cells were resuspended in a Na^+-containing buffer (not shown). In the presence of 137 mM KCl isolated hepatocytes swell whereas the same concentration of LiCl and choline chloride had no effect on cell volume. Swollen cells were not stained by trypan blue which indicates that hepatocytes remain viable in KCl-Tyrode buffer. KCl is probably entering the cell in sufficient amounts to cause osmotic water influx.

When NaCl was present in the incubation buffer in concentrations exeeding 24 mM no inhibition by Li^+ and choline$^+$ was seen on phalloidin uptake (Fig. 2). 24 mM Na^+ in the incubation buffer is regarded essential for full phalloidin transport. In contrast to Na^+ divalent cations did not affect

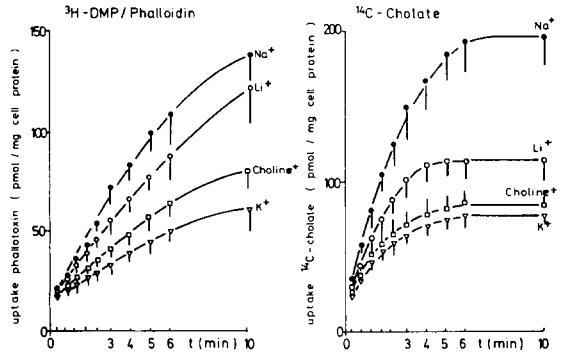

Fig. 1. Hepatocellular uptake of phalloidin and cholate in Na^+ free Tyrode buffer. $2 \cdot 10^6$ hepatocytes per ml were incubated in standard Tyrode buffer (152 mM Na^+) at 37°C under O_2/CO_2 (95:5) atmosphere. Hepatocytes were washed twice in Na^+ free Tyrode buffer and incubated for 5 min before the uptake experiment. $n = 3$ or 4. DMP, demethylphalloin.

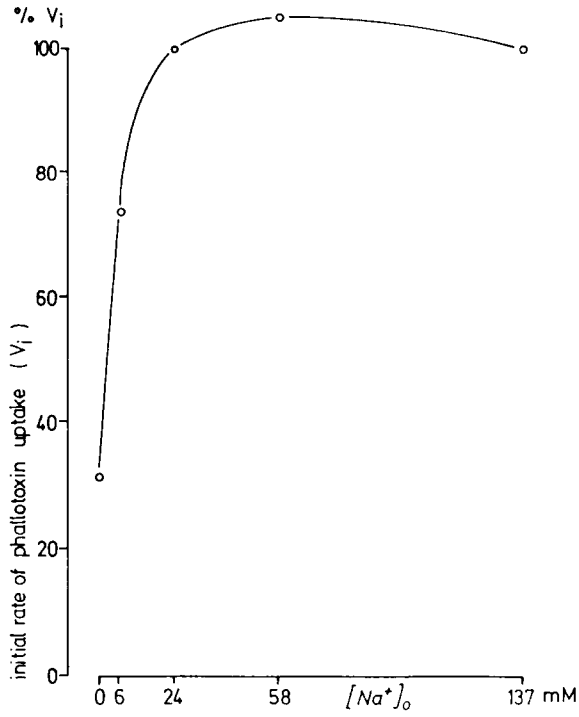

Fig. 2. Dependence of the initial rate of phalloidin uptake on the concentration of sodium in the incubation medium. Isolated hepatocytes which were incubated in Tyrode buffer were washed twice with choline chloride buffer (zero Na^+) or with the appropriate buffers by which choline chloride was substituted by 6, 24, 58 and 137 mM NaCl. After and equilibrium period of 2 min uptake of 5 μg phalloidin/70 ng demethyl[^{3}H]phalloin per ml was measured. The initial rate of uptake V_i was calculated from the Δ uptake between 15 and 75 s, 45 and 105 s, 75 and 135 s, 3 and 4 min and 4 and 5 min. Data are from a representative experiment.

neither phalloidin nor cholate uptake. Omission of external Ca^{2+} and Mg^{2+} and also chelation of membrane bound Ca^{2+} and Mg^{2+} by EGTA did neither modify the uptake of phalloidin nor of cholate (not shown).

Inhibition of phalloidin uptake by ouabain

About 50% of the transmembrane sodium fluxes are pumped by $(Na^+ + K^+)$-ATPase on kidney tubules [18,19] and human red cells [20]. This enzyme becomes completely blocked by 1 mM ouabain in isolated rat liver cells [21]. At the same concentration phalloidin uptake was inhibited within the first two minutes to about 40% (Fig. 3).

Inhibition of phalloidin and cholate uptake by monensin and nigericin

Perturbation of the sodium gradient was achieved in addition to ouabain by the Na^+-selective ionophores monensin and nigericin. Monensin permits Na^+-H^+ exchange [22] while nigericin induces Na^+-K^+ exchange in living cells [23]. Because ion exchange is electroneutral, both ionophores do not alter primarily the membrane potential but are able to dissipate a sodium gradient and should inhibit Na^+-dependent phalloidin and cholate transport. Monensin (10 μg/ml) and nigericin (1 μg/ml) blocked phalloidin and cholate uptake. When added to liver cells during an uptake experiment the ionophores switched off further hepatocellular uptake of both substrates (Fig. 4).

Because monensin catalyses exchange of Na^+ with H^+ and dissipates therefore a sodium and a proton gradient as well we tested whether a proton gradient is involved in phalloidin and cholate uptake. Acidification of liver cells with 10 mM NH_4Cl was without any effect on the uptake, 100 mM ammoniumchloride inhibited slightly phalloidin but not cholate uptake (data not shown).

Inhibition of phalloidin and cholate uptake by various gramicidins

Gramicidins are channel creating ionophores which permit the passage of Na^+, K^+, Li^+, H^+ and Cl^- [24,25] but not of Ca^{2+} [26]. By dissipation of several ion gradients gramicidins disturb the membrane potential directly. The effect of seven different gramicidins on phalloidin and cholate uptake is shown in Table I. Gramicidin D was the weakest inhibitor, valine-gramicidin A (No. 4) and the methionine-gramicidin A (No. 5) were the most potent ones. These latter gramicidins at concentrations of 10 μg/ml inhibited the initial rate (V_i) of phalloidin uptake by 83–88% and of cholate uptake by 61–69%. The effect on cholate

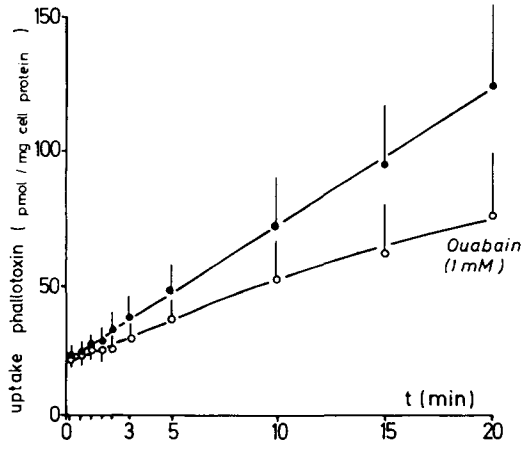

Fig. 3. Inhibition by ouabain of phallotoxin uptake. $2 \cdot 10^6$ hepatocytes/ml were incubated 30 min in the presence of 1 mM ouabain. Uptake was measured with 35 nmol/l demethyl[^{3}H]phalloin mixed with 6 μmol/l phalloidin, $n = 5$.

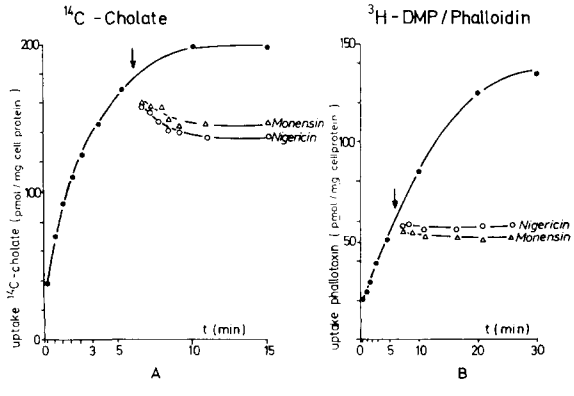

Fig. 4. Monensin and nigericin induced inhibition of phalloidin and cholate uptake in isolated rat hepatocytes. 4 ml liver cell suspension ($2 \cdot 10^6$ cells/ml) were incubated in the presence of 1 μM [^{14}C]cholate (A) and 35nM demethyl[^{3}H]phalloin/6 μM phalloidin (B). After 5 min the cell suspension was splitted. One part received at 6 min (arrow) 10 μg monensin/ml ($\triangle$) or 1 μg nigericin/ml (O). Data are from a representative experiment.

TABLE I

INHIBITORY STRENGTH OF VARIOUS GRAMICIDINS FOR THE UPTAKE OF PHALLOTOXIN AND BILE ACIDS INTO HEPATOCYTES

Isolated rat hepatocytes were incubated for 10 min in the presence of 10 μg/ml of each of the gramicidins prior to 1 μM [^{14}C]cholate or 35 nM demethyl[^{3}H]phalloin/6 μM phalloidin. The uptake was measured during 10 min for cholate and 20 min for phalloidin. For the calculation of the inhibitory potency of the gramicidins the initial part of uptake is shown in the table. $P \leq 0.05$ is regarded significant.

Gramicidin (10 μg/ml)	Uptake phallotoxin ($n = 5$)			Uptake cholate ($n = 6$)		
	nmol/mg cell protein Δ15–180 s	%	P	nmol/mg cell protein Δ15–75 s	%	P
Control (plus ethanol)	23.48 ± 6.79	100		34.42 ± 8.96	100	
D	20.32 ± 9.05	86	0.561	27.99 ± 7.71	81	0.184
6	12.63 ± 4.67	54	0.019	25.12 ± 7.10	73	0.073
7	7.48 ± 6.93	32	0.006	19.11 ± 3.67	56	0.017
1	6.76 ± 2.41	29	0.00084	13.87 ± 4.19	40	0.007
2	5.87 ± 2.47	25	0.00061	19.31 ± 5.44	56	0.020
4	3.92 ± 2.59	17	0.00102	13.50 ± 3.87	39	0.0068
5	2.92 ± 2.47	12	0.00016	10.65 ± 2.35	31	0.0035

Inhibitory potency of several gramicidins for the uptake of:
Phallotoxin: 5 > 4 > 2 ≥ 1 ≥ 7 > 6 > D
Cholate: 5 > 4 ≥ 1 > 2 ≥ 7 > 6 > D

uptake was less pronounced because this bile acid is partly taken up by a mechanism not coupled with ion gradients. This part amounted 25–30% of the total uptake. According to their inhibitory potency the most effective gramicidins for phalloidin uptake were 5 > 4 > 2 ≥ 1 ≥ 7 > 6 > D and for cholate uptake 5 > 4 ≥ 1 > 2 ≥ 7 > 6 > D (Table I).

Reduction of hepatocellular ATP content by ionophores

Phalloidin and cholate uptake was shown to be ATP dependent and could be reduced metabolic inhibitors [27]. The effect of ionophores on ATP content of isolated rat hepatocytes is listed in Table II. With exception of valinomycin, the ATP content was not reduced significantly by gramicidin and monensin. Because of technical reasons (see Methods) the number of liver cells per ml was ten times that used in uptake experiments. The concentration of the ionophores was increased 10-fold as well. 0.1 μg valinomycin/ml reduced the hepatocellular ATP level by 55%, 1.0 μg/ml by 90% within 30 s. It can be concluded therefore that

TABLE II

ATP CONTENT OF ISOLATED RAT HEPATOCYTES AFTER TREATMENT WITH IONOPHORES

1 ml cell suspension containing $20 \cdot 10^6$ hepatocytes/ml was incubated 30 s in the presence of monensin and valinomycin and 10 min in the presence of gramicidin derivative 7. Cells were carefully washed twice with fresh Tyrode buffer and boiled for 2 min in 9 mM perchloric acid. After storage in liquid nitrogen ATP was measured by means of the hexokinase/glucose-6-phosphate dehydrogenase test [16].

Control (μmol ATP/g cell protein) $n = 10$	ATP (μmol/g cell protein)					
	Gramicidin 7		Monensin		Valinomycin	
	10 μg/ml $n = 5$	100 μg/ml $n = 7$	10 μg/ml $n = 4$	100 μg/ml $n = 4$	0.1 μg/ml $n = 4$	1 μg/ml $n = 5$
4.01 ± 1.42	4.45 ± 0.78	3.47 ± 1.39	3.71 ± 0.39	3.74 ± 0.87	2.25 ± 0.4 *	0.38 ± 0.27 *

* Significant by Student's t-test with $P \leq 0.05$.

the tested ionophores except valinomycin inhibited phalloidin and cholate uptake by mechanisms unlike effects of metabolic inhibitors.

Influence of SCN^-, NO_3^- and SO_4^{2-} on phalloidin and cholate uptake

The results obtained with gramicidins indicated a membrane potential dependence of phalloidin and cholate transport. However, in these experiments the driving force resulting from the sodium gradient is altered as well. It was intended to change the membrane potential withouth alteration of the sodium concentration gradient. The lipophilic anions SCN^- and NO_3^- induce a diffusion potential which is more negative inside as compared with the Cl^- distribution potential. The entry of a positively charged substrate-Na^+ complex is facilitaed by these anions [28].

By substitution of Cl^- by SCN^- or by NO_3^- phalloidin uptake was enhanced (Fig. 5). In the presence of SO_4^{2-} both phalloidin- and cholate uptake decreased. Inhibition was also seen when the uptake was measured in the presence of 100 μM furosemide (Table III). Furosemide inhibited cholate uptake either in the presence or in the absence of Cl^- (data not shown).

Membrane potential and phalloidin uptake

The above results clearly showed that manoeuvres which altered the sodium gradient as well as the membrane potential modified phalloidin and cholate uptake. Because at zero sodium in the incubation buffer the residual phalloidin uptake was about 40% of that at 137 mM Na^+ it was concluded that this residual uptake might be driven by the hepatocellular membrane potential. In order to test the effect of a gradual membrane depolarization the external KCl concentration was increased step wise. Fig. 6 shows the effect of K^+-induced depolarization on the uptake of phalloidin and cholate. The uptake of phalloidin was stimulated to 10% in the presence of 5–10 mM

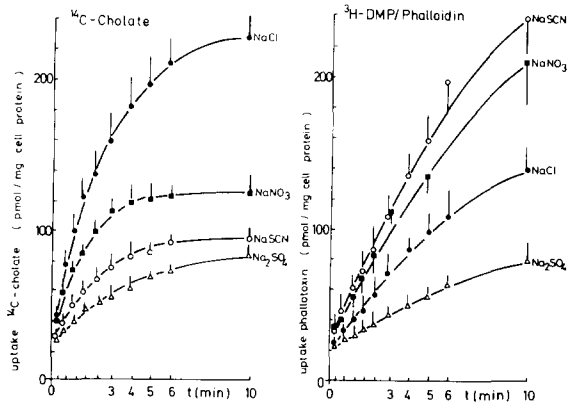

Fig. 5. Effect of Cl^- substitution on the uptake of cholate and phalloidin in isolated hepatocytes. Liver cells were incubated in standard Tyrode buffer with 137 mM NaCl at 37°C under O_2/CO_2 (95:5) atmosphere. The cells were washed twice and incubated for 5 min in the appropriate Cl^--substituted Tyrode buffer, $n = 4$. ^{3}H-DMP, demethyl[^{3}H]phalloin.

TABLE III

INHIBITION BY FUROSEMIDE ON PHALLOIDIN AND CHOLATE UPTAKE IN ISOLATED RAT HEPATOCYTES

$2 \cdot 10^6$ hepatocytes/ml were incubated in presence of 100 μM furosemide 30 s prior to uptake measurement. Phallotoxin concentration was 70 nM demethyl[^{3}H]phalloin/6 μM phalloidin. [^{14}C]Cholate concentration was 1 μM. Uptake was measured during 30 min as described in Methods. Probability values $P \leq 0.05$ were regarded significant.

	Control	In presence of 100 μM furosemide	P
Phallotoxin			
(pmol/mg cell protein)			
ΔUptake of phallotoxin between 15 and 75 s	11 ± 0.7	3 ± 1	0.00048
ΔUptake within 30 min	152 ± 28	75 ± 10	0.011
Cholate			
(pmol/mg cell protein)			
ΔUptake 15–75 s	46 ± 9	28 ± 6	0.046
ΔUptake within 30 min	178 ± 41	130 ± 31	0.182

KCl and was inhibited at higher K^+- concentrations. At 137 mM KCl (12 mM NaCl) the uptake of phalloidin was reduced to 45% and the uptake of cholate to 55% within 10 min (Fig. 6).

While depolarization caused uptake inhibition hyperpolarization might accelerate phalloidin and cholate entry into hepatocytes. In liver cells the resting membrane potential is between -30 and -50 mV [29] resembling a chloride distribution potential. In the presence of a K^+-selective ionophore, however, the membrane potential will approximate the K^+-distribution potential which is more negative and according to the Nernst equation $E_{K^+} = \ln ([K^+]_o/[K^+]_i) \cdot (RT/F)$ is about -90 to -110 mV. Valinomycin was used to induce membrane hyperpolarization thereby accelerating Na^+ influx as well as coupled phalloidin and cholate influx. However, valinomycin inhibited the uptake for both substrates at all concentrations (Fig. 7). Preincubation of hepatocytes for 30 s with 0.01 μg/ml of valinomycin reduced phalloidin uptake by 30–40%. The inhibition was not markedly enhanced by a longer incubation period (Fig. 7A). Higher concentration of valinomycin blocked cholate and phalloidin uptake completely (Fig. 7B). Kinetics of K^+ efflux induced by valinomycin was measured by flame photometry (not shown). In presence of valinomycin concentrations which were comparable to those mentioned above (2 μg/20 · 10^6 liver cells) 18% of hepatocellular

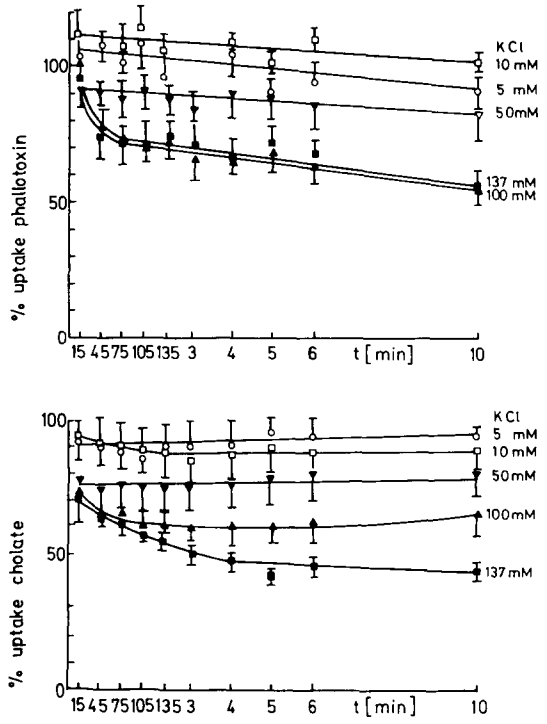

Fig. 6. Uptake of phalloidin and cholate into hepatocytes after depolarization induced by reversed electrochemical K^+ gradient. Isolated hepatocytes were incubated 5 min before addition of 1 μM [^{14}C]cholate and 35 nM demethyl[^{3}H]phalloin /6 μM phalloidin in the following buffer media: (○) Tyrode buffer with 5 mM KCl/132 mM NaCl, (□) 10 mM KCl/127 mM NaCl, (▽) 50 mM KCl/87 mM NaCl, (▲) 100 mM KCl/37 mM NaCl, (■) 137 mM KCl/0 mM NaCl. The values are expressed as percent of control uptake, which was the uptake in the presence of the standard Tyrode buffer with 2.7 mM KCl/137 mM NaCl. $n = 4$.

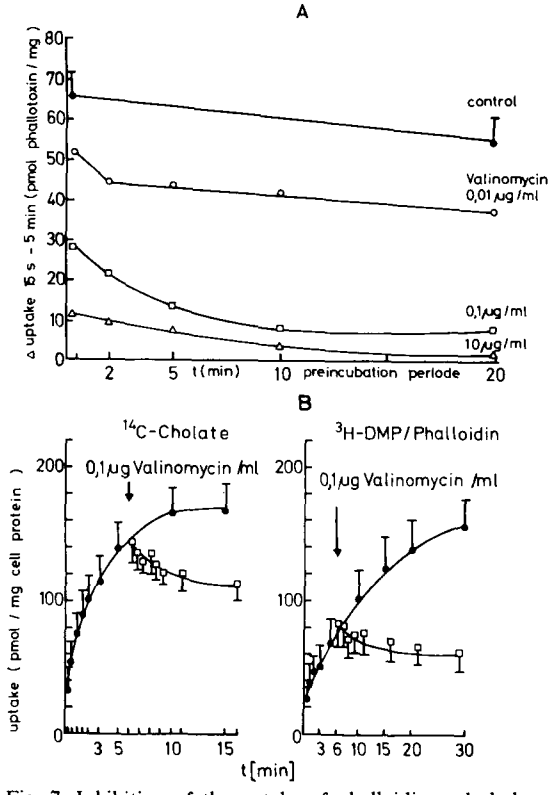

Fig. 7. Inhibition of the uptake of phalloidin and cholate by valinomycin. (A) Time- and concentration-dependence of the valinomycin effect. Isolated hepatocytes were preincubated in the presence of (○) 0.01 μg/ml, (□) 0.1 μg/ml, (△) 10.0 μg/ml valinomycin during 15 s, 2 min, 5 min, 10 min and 20 min before phalloidin uptake was measured. $n = 5$. (B) Inhibition of the uptake of phalloidin and cholate by 0.1 μg valinomycin. ^{3}H-DMP, demethyl[^{3}H]phalloin.

potassium was released during 20 min; half of that K^+ leaked out within 2 min. The added valinomycin was completely trapped by hepatocytes. Incubation of fresh liver cells in the supernatant of already treated cells caused not further K^+ leakage.

Discussion

The importance of secondary active cotransport for the accumulation of substrates in cells is widely recognized and several reviews deal with the description and characterization of such transport systems [17,30–34]. We find a Na^+-cotransport for phalloidin which exhibits all characteristics of the sodium cotransport system for bile acids. Besides the sodium gradient the membrane potential is an additional force for both phalloidin and cholate uptake.

Arguments which satisfy the accepted criteria of a sodium dependence are:
(1) Substitution of extracellular Na^+ by K^+, Li^+ or choline$^+$ reduced phalloidin uptake (Fig. 1).
(2) Inhibition of the $(Na^+ + K^+)$-ATPase by ouabain inhibited the phalloidin uptake (Fig. 3).
(3) Dissipation of the sodium gradient by the carboxylic carrier ionophores monensin and nigericin inhibited phalloidin uptake (Fig. 4).

However, the effect of sodium substitution depends upon the kind of substituent: The inhibition of phalloidin and cholate uptake was most effective by K^+ and least effective by Li^+, whereas choline was moderately effective. The pronounced inhibition by K^+ might be due to side effects on membrane potential and cell volume. The latter was altered in presence of 137 mM KCl ($[Na^+]_o$ = 0 mM) but remained unchanged by 100 mM KCl (and 49 mM $[NaCl]_o$), which is in agreement with earlier experiments [35]. Li^+ was insufficient in substituting the driving Na^+ gradient. Only weak inhibition was found on phalloidin uptake whereas cholate uptake was inhibited markedly. However, choline chloride inhibited both transports to a similar degree (Fig. 1) and was, therefore, used in all further experiments for replacement of NaCl.

The rate of phalloidin uptake as well as of cholate uptake [2] depends on the extracellular Na^+ concentration. Na^+ concentration in the incubation buffer of 24 mM was sufficient to establish fully phalloidin uptake (Fig. 2) 24 mM, however, is precisely the concentration of intracellular sodium of liver cells [36]. Therefore only Na^+ concentrations > 24 mM generate an inward directed Na^+ gradient and can energenize Na^+-dependent phalloidin uptake into liver cells. Below 24 mM Na^+ phalloidin uptake is probably maintained by the membrane potential. Several results argue for the assumption of a membrane potential dependent uptake of phalloidin and cholate.

Depolarization of liver cells by a reversed potassium gradient inhibited phalloidin and cholate uptake (Fig. 6).

Dissipation of the membrane potential by gramicidins blocked phalloidin and cholate uptake (Table I).

Hyperpolarization by lipophilic SCN^- and NO_3^- anions stimulated phalloidin but reduced cholate uptake (Fig. 5).

Modification of the membrane potential by the potassium ionophore valinomycin blocked phalloidin and cholate uptake (Fig. 7).

For phalloidin and cholate uptake was reduced by half, presumably the two driving forces contribute to equal amounts to the energenization of this transport.

Lipophilic anions were found to stimulate the carrier mediated passage of positively charged compounds through membranes [28]. For phalloidin a 100 percent enhanced uptake rate was observed (Fig. 5). The probable interpretation is to assume the permeation of a positively charged phalloidin-sodium aggregate. Its uptake is accelerated in the presence of SCN^- and NO_3^- by the internal negative membrane potential. The observed stimulation indicates that the coupling ratio between phalloidin and Na^+ could be equimolar because phalloidin bears no negative charge. Uptake of phalloidin in terms of charged ion substrate units is regarded to be electrogenic. In accordance to this hypothesis the hydrophilic anion SO_4^{2-} inhibited phalloidin uptake.

In contrast to phalloidin, cholate uptake was inhibited under identical conditions (Fig. 5). Cholate uptake was Cl^- dependent. Substitution of chloride either by SO_4^{2-}, NO_3^- or SCN^- reduced uptake. A satisfactory explanation may be that Cl^- is selectively needed in bile acid uptake for electroneutrality reasons. If one assumes a cou-

pling ratio between Na^+ and cholate of 2 : 1, which was reported already [2], Cl^- may serve as discharging counteranion. Thus the bile acid carrier might have distinct binding sites for two sodium, one bile acid and one chloride anion. With respect to phalloidin the cyclopeptide takes seat on the same carrier and one negative bile acid charge is now substituted by a neutral molecule. Phalloidin/Na^+/carrier units remain positively charged. This may explain why phalloidin uptake differed from cholate uptake with respect to Cl^- dependency but was very much sensitive to changes of the membrane potential by lipophilic anions.

Our results on cholate uptake differ from findings about taurocholate uptake into membrane vesicles from rat liver cells [4,5]. In the vesicle model $NaNO_3$ stimulated bile acid uptake. The discrepancy may either result from different transports for both bile acids or from a different behaviour of bile acid uptake in isolated hepatocytes and membranes vesicles.

Indeed hepatocytes do not respond like membrane vesicles as was seen in the experiments with valinomycin. Although the above results suggest an electrogenic phalloidin uptake, valinomycin caused no overshoot uptake. Such transient stimulation was found on brush border membrane vesicles during electrogenic taurocholate uptake [37]. Valinomycin inhibited both phalloidin and cholate uptake on isolated hepatocytes (Fig. 7) despite K^+ outflow at comparable concentrations. The reason why the supposed electrogenic phalloidin uptake did not respond to valinomycin (but to NO_3^- and SCN^-) by an overshoot might be that simultanously with K^+ leakage a rapid loss of ATP occurred (Table II). If ATP is consumed for the movement of counterions, opposing valinomycin-induced potassium outleak, the K^+-induced hyperpolarization is suppressed. The effect of valinomycin on living cells might therefore be different from its effects on electrogenic uptake by non metabolizing membrane vesicles.

Both, sodium ion gradient and membrane potential could well explain the previously shown accumulation of free, nonbound phalloidin into liver cells [27]. The complexity of the driving forces, however, implies that drugs which inhibit phalloidin uptake can do so by combined interactions, e.g., by depletion of cell ATP, by dissipation of ion gradients, by destruction of $\Delta\psi$ and by direct interaction with the transport proteins. Direct interaction was already shown for trypsin, cholate, iopodate, DIDS and several affinity labelled bile acid (for references, see Ref. 10) and might be the reason for transport inhibition by furosemide. The latter blocked cholate and phalloidin uptake in presence and absence of chloride which indicates a mechanism of inhibition which is different from its effect on chloride-coupled sodium transport. Because of the various possibilities of drug interaction, phalloidin poisoning cannot be regarded as appropriate model to test hepatoprotective drugs.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft, grant Pe 250/3-3. We wish to express our gratitude to Professors Drs. Th. Wieland and H. Faulstich for providing demethyl[^{3}H]phalloin. We are grateful to Professor Dr. E Gross†, Bethesda for the generous gift of gramicidin A derivatives. Dr. Singh Chhatwal is acknowledged for advice in preparing the manuscript and Dr. David Ferry for support in statistics.

References

1 Reichen,J. and Paumgartner, G. (1976) Am. J. Physiol. 321, 734–740
2 Anwer, M.S. and Hegner, D. (1978) Hoppe-Seyler's Z. Physiol. Chem. 359, 181–192
3 Scharschmidt, B.F. and Stephens, J.E. (1981) Proc. Natl. Acad. Sci. USA 78, 986–990
4 Ruifrok, P.G. and Meijer, D.K.F. (1982) Liver 2, 28–34
5 Inoue, M., Kinne, R., Tran, T. and Arias, I.M. (1982) Hepatology 2, 572–579
6 Paumgartner, G. and Reichen, J. (1975) Experientia 31, 306–308
7 Anwer, M.S. and Hegner, D. (1978) Hoppe-Seyler's Z. Physiol. Chem. 359, 1027–1030
8 Minder, E. and Paumgartner, G. (1979) Experienta 35, 888–890
9 Petzinger, E., Joppen, C. and Frimmer, M. (1983) Naunyn-Schmiedeberg's Arch. Pharmacol. 322, 174–179
10 Frimmer, M. (1979) Trends Pharmacol. Sci. 3, 395–397
11 Berry, M.S. and Friend, D.S. (1969) J. Cell. Biol. 43, 506–529
12 Petzinger, E. and Seeger, R. (1976) Naunyn-Schmiedeberg's Arch. Pharmacol. 295, 211–213
13 Ziegler, K., Petzinger, E., Grundmann, E. and Frimmer, M. (1979) Naunyn-Schiedeberg's Arch. Pharmacol. 306, 295–300

14 Frimmer, M., Petzinger, E., Ziegler, K. and Veil, L.B. (1980) Naunyn-Schmiedeberg's Arch. Pharmacol. 311, 91–94
15 Klingenberg, M. and Pfaff, E. (1967) Methods Enzymol. 10, 680–684
16 Lamprecht, W. and Trautschold, I. (1970) in Methoden der enzymatischen Analyse (Bergmeyer, H.U., ed.), pp. 2024–2033, Verlag Chemie, Weinheim
17 Heinz, E. (1972) Na-linked transport of organic solutes. The coupling between electrolyte and nonelectrolyte transport in cells, Springer Verlag Berlin, Heidelberg, New York
18 Berry, C.A. and Rector, F.C. (1980) Miner. Electrolyte Metab. 4, 149–160
19 Jørgensen, P.L. (1980) Physiol. Rev. 60, 864–917
20 Glynn, I.M. (1957) J. Physiol. 136, 148–173
21 Boyer, J.L. and Reno, D. (1975) Biochim. Biophys. Acta 401, 59–72
22 Pressmann, B.C. and Fahim, M. (1982) Annu. Rev. Pharmacol. Toxicol. 22, 465–490
23 Heinz, A., Jackson, J.W., Richey, B.E. Sachs, G. and Schafer, J.A. (1981) J. Membrane Biol. 62, 149–160
24 Myers, V.B. and Haydon, D.A. (1972) Biochim. Biophys. Acta 274, 313–322
25 Bakker, E.P. (1979) in Antibiotics, Vol. 5, (Hahn, F.E., ed.), pp. 67–97, Springer-Verlag, Berlin, Heidelberg, New York
26 Hladky, S.B. and Haydon, D.A. (1972) Biochim. Biophys. Acta 274, 294–312
27 Petzinger, E. and Frimmer, M. (1982) Naunyn-Schmiedeberg's Arch. Pharmacol. 319, 87–92
28 Sacktor, B. (1982) Curr. Top. Membranes Transp. 13, 291–300
29 Wondergem, R. and Harder, D.R. (1980) J. Cell Physiol. 102, 193–197
30 Le Fevre, P.G. (1975) Curr. Top. Membranes Transp. 7, 109–215
31 Crane, R.K. (1977) Rev. Physiol. Biochem. Pharmacol. 78, 101–159
32 Heinz, E. (1978) Mechanics and Energetics of Biological Transport, Springer-Verlag, Berlin, Heidelberg, New York
33 Kilberg, M.S. (1982) J. Membrane Biol. 69, 1–12
34 Turner, R.J. (1983) J. Membrane Biol. 76, 1–15
35 Frimmer, M. and Kroker, R. (1973) Naunyn-Schmiedeberg's Arch. Pharmacol. 276, 99–102
36 Mazet, J.L., Claret, M. and Claret, B. (1974) J. Membrane Biol. 18, 335–350
37 Lücke, H., Stange, G., Kinne, R. and Murer, H. (1978) Biochem. J. 174, 951–958
38 Petzinger, E. (1981) Naunyn-Schmiedeberg's Arch. Pharmacol. 316, R 21