Effects of taurolithocholate, a Ca^{2+}-mobilizing agent, on cell Ca^{2+} in rat hepatocytes, human platelets and neuroblastoma NG108-15 cell line

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The monohydroxy bile acid taurolithocholate permeabilizes the endoplasmic reticulum to Ca^{2+} in rat liver cells. To assess whether this action on the endoplasmic reticulum was restricted to this tissue, the effects of bile acid were investigated in two cell types quite unrelated to rat hepatocyte, namely human platelets and the neuronal NG108-15 cell line. The results showed that taurolithocholate (3–100 μM) had no effect on free cytosolic $[Ca^{2+}]$ in human platelets and NG108-15 cells, whereas it increased it from 180 to 520 nM in rat hepatocytes. In contrast, in cells permeabilized by saponin, taurolithocholate initiated a profound release of the stored Ca^{2+} from the internal Ca^{2+} pools in the three cell types. The bile acid released 90% of the Ca^{2+} pools, with rate constants of about 5 min^{-1} and half-maximal effects at 15–30 μM. The results also showed that, in contrast with liver cells, which displayed an influx of [^{14}C]taurolithocholate of 2 nmol/min per mg, human platelets and the neuronal cell line appeared to be resistant to [^{14}C]taurolithocholate uptake. The influx measured in these latter cells was about 100-fold lower than in rat liver cells. Taken together, these data suggest that human platelets and NG108-15 cells do not possess the transport system for concentrating monohydroxy bile acids into cells. However, they show that human platelets and neuronal NG108-15 possess, in common with liver cells, the intracellular system responsible for taurolithocholate-mediated Ca^{2+} release from internal stores.

INTRODUCTION

Certain bile acids such as the monohydroxy bile acid taurolithocholate (EC_{50} = 20 μM) markedly alter cell Ca^{2+} metabolism in isolated rat hepatocytes. In suspensions of intact cells, taurolithocholate increases $[Ca^{2+}]_i$ and stimulates Ca^{2+} efflux (Combettes *et al.*, 1988*a*; Anwer *et al.*, 1988, 1989). Several observations indicate that this bile acid mobilizes Ca^{2+} from the InsP_3-dependent pool (Combettes *et al.*, 1988*a*,*b*). First, in intact hepatocytes the taurolithocholate-mediated $[Ca^{2+}]_i$ rise is not altered by removal of external Ca^{2+}. Second, the pool is the same as that permeabilized by the InsP_3-dependent hormone vasopressin. Third, in saponin-treated cells, taurolithocholate releases Ca^{2+} from the same pool as InsP_3 does. Though this has not been yet completely demonstrated, the effect seems to be mediated by an increase in the Ca^{2+}-permeability of the endoplasmic reticulum (ER) membrane (Combettes *et al.*, 1989). It has been also shown that, when applied to experimental animals at concentrations in the range 10–100 μM, the monohydroxy bile acids and some of their conjugates promote a major and sustained inhibition of bile secretion (Javitt, 1975; Mathis *et al.*, 1983; Oelberg *et al.*, 1984).

Since monohydroxy bile acids may be present at micromolar concentrations in portal-vein plasma under pathological conditions (Matern & Gerok, 1979), the question arises whether the ER of other cell types is the target of the Ca^{2+}-permeabilizing action of these molecules. To assess this point, Ca^{2+} responses to taurolithocholate in rat hepatocytes were compared with those displayed by human platelets (Rink & Sage, 1990) and the neuroblastoma NG108-15 cell line (Hamprecht, 1977; Nirenberg *et al.*, 1983). These cells are unrelated to the liver and possess the InsP_3-dependent Ca^{2+} pool which could be the potential target of the action of bile acids (Agranoff *et al.*, 1983; Reiser & Hamprecht, 1985; Higashida *et al.*, 1986; Jackson *et al.*, 1987; Rink & Sage, 1990). The results show that taurolithocholate fails to affect $[Ca^{2+}]_i$ in intact human platelets and the neuroblastoma NG108-15 cell line. In contrast, prior permeabilization of the plasma membrane by saponin allows taurolithocholate to release Ca^{2+} from the hormone-sensitive pool. The results also show that the absence of effect of taurolithocholate results from a primary lack of a transport system for bile acids in the plasma membrane of these cells.

MATERIALS AND METHODS

Materials

$^{45}Ca^{2+}$ and Ins(1,4,5)P_3 were obtained from Amersham, and [^{14}C]taurolithocholate was from Mallinckrodt G.m.b.H. or was synthesized by C.S. Gelatin was obtained from DIFCO, quin2, fura2/AM and quin2/AM were from Lancaster Synthesis and Molecular Probes, ionomycin and hirudin were from Calbiochem, and collagenase was from Boehringer. All other chemicals were purchased from Sigma and were of the highest purity commercially available.

Preparation of rat hepatocytes

Hepatocytes were prepared as described by Combettes *et al.*

Abbreviations used: $[Ca^{2+}]_i$, concentration of free cytosolic Ca^{2+}; EC_{50}, concn. giving 50% of maximal effect; ER, endoplasmic reticulum; quin2, free tetra-anion of quin2/AM; quin2/AM, quin2 tetra-acetoxymethyl ester; fura2, free tetra-anion of fura2/AM; fura2/AM, fura2 tetra-acetoxymethyl ester; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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(1988*a*). They were isolated from female Wistar rats and maintained (2×10^6/ml) in an Eagle's medium (EML) containing 116 mM-NaCl, 5.4 mM-KCl, 1.2 mM-$CaCl_2$, 1.2 mM-$MgCl_2$, 0.92 mM-NaH_2PO_4, 25 mM-$NaHCO_3$, 15 mg of gelatin/ml, 5.6 mM-glucose and vitamins and amino acids. It was gassed with O_2/CO_2 (19:1) (pH 7.4) at 37 °C. Cell viability as estimated by Trypan Blue exclusion was always greater than 98% and remained stable for 4–5 h.

Measurement of $[Ca^{2+}]_i$ in rat hepatocytes

A 2 ml portion of cells (0.4×10^6/ml) was loaded with 50 μM-quin2/AM in EML for 150 s, and then washed once by centrifugation at 50 *g* for 1 min in the same medium lacking vitamins, amino acids and gelatin as described previously (Combettes *et al.*, 1988*a*). The cell pellet was resuspended in the same medium and transferred to the spectrofluorimeter cuvette under continuous gassing and stirring at 37 °C. The $[Ca^{2+}]_i$ was calibrated and corrected for autofluorescence.

Ca^{2+} release from saponin-treated rat liver cells

Hepatocytes (2×10^6/ml) were incubated in an internal medium (IML) and permeabilized by saponin as reported by Combettes *et al.* (1989). In brief, the medium contained 100 mM-KCl, 20 mM-NaCl, 5 mM-$MgCl_2$, 0.96 mM-NaH_2PO_4 and 25 mM-Hepes (pH 7.15) at 37 °C. It also contained 5 μM of the mitochondrial uncoupler CCCP, 1.5 mM-ATP, 5 mM-phosphocreatine and 5 units of creatine kinase/ml, and 10 μM-quin2. Samples of cell suspension (2 ml) were transferred to the spectrofluorimeter cuvette. The Ca^{2+} uptake was initiated by adding 50 μg of saponin/ml. The contaminant Ca^{2+} was 3–4 μM as measured by atomic-absorption spectroscopy. The cell Ca^{2+} content represents the amount of Ca^{2+} sequestered within the internal pool and totally mobilized by 5 μM-ionomycin. It was calculated from the difference between the Ca^{2+} bound to quin2 before and after addition of ionomycin and expressed as nmol of Ca^{2+}/mg of cell protein.

Influx and uptake of bile hepatocytes

This was performed as reported by Combettes *et al.* (1990). A 2 ml portion of cells (2×10^6/ml) was incubated in EML at 37 °C. [^{14}C]Taurolithocholate uptake was initiated by adding the labelled bile acid (0.05 μCi/ml). Cell samples (100 μl) were transferred to ice-cold Eppendorf tubes containing 200 μl of dibutyl phthalate ($d = 1.045$) and 1 ml of a washing solution containing 144 mM-NaCl, 5 mM-$CaCl_2$ and 5 mM-Tris/HCl (pH 7.4), supplemented with 2% BSA used as a bile acid chelator to decrease maximally the non-intracellular bound fraction. The tubes were centrifuged (10000 *g*, 2 min) and the pellets counted for radioactivity in a scintillation spectrometer.

Preparation of isolated human platelets

Human platelets were prepared as reported by Hallam & Rink (1985). Samples (20 ml) of freshly drawn blood from healthy volunteers were added to 3.3 ml of acid–citrate–dextrose anticoagulant in plastic test tubes. The anticoagulant contained 25 mg of trisodium citrate, 15 mg of citric acid and 20 mg of dextrose per ml, so that the final whole-blood citrate concentration was 22 mM at pH 6.5. The anticoagulated blood was centrifuged at 700 *g* at room temperature for 5 min, and the platelet-rich plasma was kept at room temperature.

Measurement of $[Ca^{2+}]_i$ in platelets

Platelets were loaded by incubating the platelet-rich plasma with 3 μM-fura2/AM for 45 min at 37 °C. Cells were then sedimented at 350 *g* for 20 min, and the pellet was resuspended in 8–10 ml of a medium (MP1) containing 145 mM-NaCl, 5 mM-KCl, 1 mM-$MgSO_4$, 10 mM-dextrose, 0.05 unit of hirudin/ml, 20 μg of apyrase/ml and 10 mM-Hepes (pH 7.4). The resulting suspension, containing typically $(1.2–1.9) \times 10^8$ cells/ml, was kept at 20 °C in stoppered plastic tubes. Before experiments, portions of the suspension were supplemented with 1 mM-$CaCl_2$. $[Ca^{2+}]_i$ was measured as indicated in Pollock & Rink (1986). Samples (0.8 ml) of platelet preparations were transferred into the spectrofluorimeter cuvette thermostatically maintained at 37 °C and magnetically stirred. In a few experiments, ionomycin was used to demonstrate the presence of Ca^{2+}-loaded internal pools in human platelets. Therefore, 2.1 mM-EGTA was added to the 1 mM-Ca^{2+} medium 30 s before the ionophore to lower external free Ca^{2+} to 200 nM as calculated from the dissociation constant for the EGTA–Ca complex of 220 nM in this medium. At the end of the experiments, $[Ca^{2+}]_i$ was corrected for autofluorescence.

Ca^{2+} release in saponin-treated human platelets

Human platelets were permeabilized essentially as indicated in Yoshida *et al.* (1988). The platelet-rich plasma was centrifuged at 350 *g* for 20 min, and the cells were resuspended in the same volume of medium (MP2) containing 140 mM-NaCl, 2.7 mM-KCl, 5.5 mM-glucose, 10 mM-Pipes buffer (pH 6.5) and 0.1 mg of apyrase/ml at 20 °C. The cells were centrifuged at 480 *g* for 10 min and resuspended in a volume of the internal medium (IMP) required to give 3.6×10^8 cells/ml. The IMP medium contained 100 mM-KCl, 20 mM-NaCl, 5 mM-$MgCl_2$, 1 mM-NaH_2PO_4, 0.625 mM-$CaCl_2$ and 1 mM-EGTA (final free $[Ca^{2+}] = 0.5$ μM), 3 mM-ATP, 5 mM-phosphocreatine, 5 units of creatine kinase/ml, 2.5 μM-CCCP, 5 μM-oligomycin and 25 mM-Hepes (pH 7.15). Platelets were permeabilized with 15 μg of saponin/ml and loaded to equilibrium (20 min) with 2 μCi of $^{45}Ca^{2+}$. $^{45}Ca^{2+}$ release was stopped at intervals by diluting cell samples (50 μl) with 5 ml of the ice-cold IMP lacking $CaCl_2$ and supplemented with 0.5 mM-EGTA. The suspension was immediately vacuum-filtered through GF/C filters, then washed twice further with 5 ml of the same ice-cold solution. Filters were immediately removed and counted for radioactivity by liquid-scintillation counting.

[^{14}C]Taurolithocholate uptake by human platelets

Cells (6×10^8/ml) were suspended in the saline incubation medium MP2 used for measuring $[Ca^{2+}]_i$. Taurolithocholate and [^{14}C]taurolithocholate (0.05 μCi/ml) were added to 1 ml samples of the cell suspension at 37 °C. At appropriate times, 130 μl samples were removed and transferred into test tubes containing 2 ml of the same ice-cold medium supplemented with 20 mg of albumin/ml, then centrifuged and washed twice at 4200 *g* for 5 min. The pellet was dispersed in a solution containing 30 mg of SDS/ml and 0.5 M-Tris (pH 7.5) and warmed at 37 °C to complete solubilization. The radioactivity was counted as for the liver cells.

Culture of neuroblastoma NG108-15 cell line

Clonal mouse neuroblastoma × glioma hybrid NG108-15 cells were grown in Dulbecco's modified Eagle's medium containing 50 mg of fetal-calf serum, 50 units of penicillin and 50 μg of streptomycin per ml at 37 °C in air/CO_2 (19:1) essentially as described by Jourdon *et al.* (1986). Cells (1.8×10^5) were plated out on sterile 100 mm × 15 mm plastic dishes and allowed to grow to confluence for 4 days before use [$(2–3) \times 10^6$ cells/dish].

Measurement of $[Ca^{2+}]_i$ in neuroblastoma NG108-15 cell line

Cells were loaded with fura2 and $[Ca^{2+}]_i$ was measured as indicated in Jackson *et al.* (1987). They were removed from culture dishes by incubation for 10 min at 37 °C in a medium

containing 137 mM-NaCl, 5.4 mM-KCl, 0.17 mM-Na_2HPO_4, 0.22 mM-KH_2PO_4, 55 mM-sucrose and 5.5 mM-glucose (pH 6.8). The resulting cell suspension was centrifuged at 50 *g* for 1 min, and then the pellet was resuspended in the incubation medium (MN), containing 154 mM-NaCl, 5.6 mM-KCl, 1.8 mM-$CaCl_2$, 1.2 mM-$MgCl_2$, 1.2 mM-NaH_2PO_4 and 10 mM-Hepes (pH 7.4). For dye loading, this medium was supplemented with 2 μM-fura2/AM, 3 mM-probenecid to decrease fura2 leakage from the cells and 2 mg of BSA/ml. A 2 ml portion of cells (1.5×10^6/ml) was incubated in this loading medium for 45 min at 37 °C. At the end of the loading period, cells were washed once by centrifugation at 50 *g* for 1 min, resuspended in MN and transferred in the thermostatically regulated gently agitated cuvette of the spectrofluorimeter. When ionomycin was used to demonstrate the presence of Ca^{2+}-loaded internal pools in neuroblastoma cells, 3.8 mM-EGTA was added to the 1.8 mM-Ca^{2+} medium 30 s before the ionophore to lower external free Ca^{2+} to 200 nM. At the end of the experiment, the $[Ca^{2+}]_i$ was corrected for autofluorescence.

Ca^{2+} release in saponin-treated neuroblastoma NG108-15 cell line

The cells were permeabilized with saponin by the procedure developed by Gill & Chueh (1985) for the parent neuroblastoma N1E-15 cell line. A 2 ml portion of cells incubated in MN medium was sedimented at 600 *g* for 1 min, then resuspended in the internal-like medium (IMN), comprising 140 mM-KCl, 10 mM-NaCl, 2.5 mM-$MgCl_2$, 10 mM-Hepes (pH 7.0) and 50 μg of saponin/ml, for 10 min at 37 °C. The detergent was removed by centrifuging cells at 600 *g* for 1 min and resuspending them in IMN lacking saponin but supplemented with 1.5 mM-ATP, 5 mM-phosphocreatine and 5 units of creatine kinase/ml, 500 μM-$CaCl_2$, 850 μM-EGTA (final free $[Ca^{2+}]$ = 300 nM), 5 μM-CCCP and traces of $^{45}Ca^{2+}$ (2 μCi/ml). The cells (0.5×10^6 cells/ml) were allowed to take up $^{45}Ca^{2+}$ to equilibrium (20 min). At the times indicated in the relevant Figure, 500 μl samples were rapidly transferred to tubes containing ice-cold medium containing 144 mM-NaCl, 5 mM-Tris (pH 7.4) and an excess of 5 mM-$CaCl_2$ to accelerate dissociation of the non-specific $^{45}Ca^{2+}$ and the contaminant $^{45}Ca^{2+}$. The tubes were vacuum-filtered through Whatman GF/C filters, then washed further with 3×5 ml of the ice-cold medium. Filters were counted for radioactivity as indicated for human platelets.

[^{14}C]Taurolithocholate uptake by neuroblastoma NG108-15 cell line

A 2 ml portion of cells [$(2–3) \times 10^6$/ml] was incubated in the incubation medium (MN) lacking probenecid and BSA but supplemented with different concentrations of taurolithocholate and 0.05 μCi of [^{14}C]taurolithocholate/ml at 37 °C. At the times indicated in Fig. 5, samples of cells (130 μl) were removed and transferred to tubes containing 2 ml of the ice-cold incubation medium supplemented with 2% BSA (1–4 °C). The cells were centrifuged and washed twice at 50 *g* for 1 min. The resulting pellet was dispersed in 110 μl of water and the radioactivity counted after addition of the appropriate scintillant.

RESULTS

Effects of taurolithocholate on intact human platelets and neuroblastoma NG108-15 cell line

It has been shown that taurolithocholate markedly increases $[Ca^{2+}]_i$ and stimulates Ca^{2+} efflux from intact rat hepatocytes (Combettes *et al.*, 1988*a*; Anwer *et al.*, 1988, 1989). This results from a transient permeabilization of the ER to Ca^{2+}. This effect in intact hepatocytes loaded with quin2 is shown in Fig. 1(*a*). The cells showed a rapid rise in $[Ca^{2+}]_i$ from 185 ± 5 nM to 524 ± 53 nM ($n = 8$) within 20 s in response to a maximal concentration of the bile acid (100 μM). The cells also responded to a maximally effective concentration (10 nM) of the $InsP_3$-dependent hormone vasopressin, which increased $[Ca^{2+}]_i$ from 202 ± 40 nM to 925 ± 25 nM ($n = 4$). However, the response to vasopressin was more sustained, owing to the delayed contribution of the influx of external Ca^{2+}. This latter component was not increased by taurolithocholate (Combettes *et al.*, 1990). Fig. 1(*b*) shows the effect of the bile acid on the $[Ca^{2+}]_i$ of human platelets. $[Ca^{2+}]_i$ was detected with the fluorescent indicator fura2, which is a more convenient indicator than quin2 in these cells. Platelets appeared to be quite resistant to application of the same concentration of taurolithocholate. This lack of action did not result from depletion of the internal Ca^{2+} stores, as evidenced by the fact that addition of a maximal concentration of the $InsP_3$-dependent hormone thrombin (0.2 units/ml), as well as the Ca^{2+} ionophore ionomycin (5 μM applied in low-Ca^{2+} medium; see legend to Fig. 1), could release stored Ca^{2+} and promote the expected rise in $[Ca^{2+}]_i$ in the same cell sample (Pollock & Rink, 1986). The hormone increased the basal $[Ca^{2+}]_i$ from 118 ± 22 nM to 951 ± 283 nM ($n = 3$). A similar conclusion could be drawn with the neuroblastoma cell line loaded with fura2. Fig. 1(*c*) shows that these cells behave also quite differently from rat hepatocytes, being completely insensitive to 100 μM-taurolithocholate. The natural hormone bradykinin, at a maximal concentration of 10 μM, that mobilizes internal Ca^{2+} via $InsP_3$ in neuroblastoma cells (Reiser & Hamprecht, 1985; Higashida *et al.*, 1986; Jackson *et al.*, 1987), augmented $[Ca^{2+}]_i$ from 103 ± 16 nM to 261 ± 69 nM ($n = 5$). Ionomycin (5 μM) was also able to induce Ca^{2+} release, confirming the existence of fully loaded Ca^{2+} stores.

The dose–response curves for the action of taurolithocholate on $[Ca^{2+}]_i$ of the three cells are shown in Fig. 2. The lack of a taurolithocholate effect on human platelets and neuroblastoma cells was confirmed at other concentrations of the bile acid. The half-maximal effect on liver cells was found at 30 μM.

Effect of taurolithocholate on permeabilized cells

A major question that arises from the above experiments is the recognition of which step, from the uptake of extracellular taurolithocholate to release of internal Ca^{2+}, is lacking in human platelets and neuroblastoma cells. The inability of taurolithocholate to increase $[Ca^{2+}]_i$ in these cells could result from the lack of the transport system able to concentrate taurolithocholate into the cells, or from the lack of the site of action on the ER itself. To distinguish between these possibilities, the action of the bile acid was tested in saponin-treated cells. Permeabilized cells were incubated in an internal-like medium containing buffered low $[Ca^{2+}]$, inhibitors of mitochondrial Ca^{2+} uptake and ATP, which allowed optimal loading of the ER (Burgess *et al.*, 1984; Gill & Chueh, 1985; Yoshida *et al.*, 1988). In addition, the action of the bile acid was compared with that induced by $InsP_3$. Fig. 3 shows the time-courses of the Ca^{2+} releases induced by maximally effective concentrations of taurolithocholate (100 μM) and $InsP_3$ (10 μM) in the three permeabilized cells. A major observation was made: unexpectedly, the bile acid promoted a rapid and profound release of Ca^{2+} from the internal pool in platelets and neuroblastoma cells as well as in liver cells (Fig. 3). $InsP_3$ also initiated a fast release from the permeabilized cells. Taurolithocholate mobilized approx. 86–97% of the Ca^{2+} releasable by ionomycin, and this action was almost complete within 30 s. Graphical analysis of the data showed that, despite individual differences in the amounts of the Ca^{2+} accumulated by the three cells (see the legend to Fig. 3), the rate constants of taurolithocholate-mediated Ca^{2+} releases were 5.0, 5.1 and 4.3 min^{-1} for liver cells, platelets and neuroblastoma cells respectively. This rate constant is a

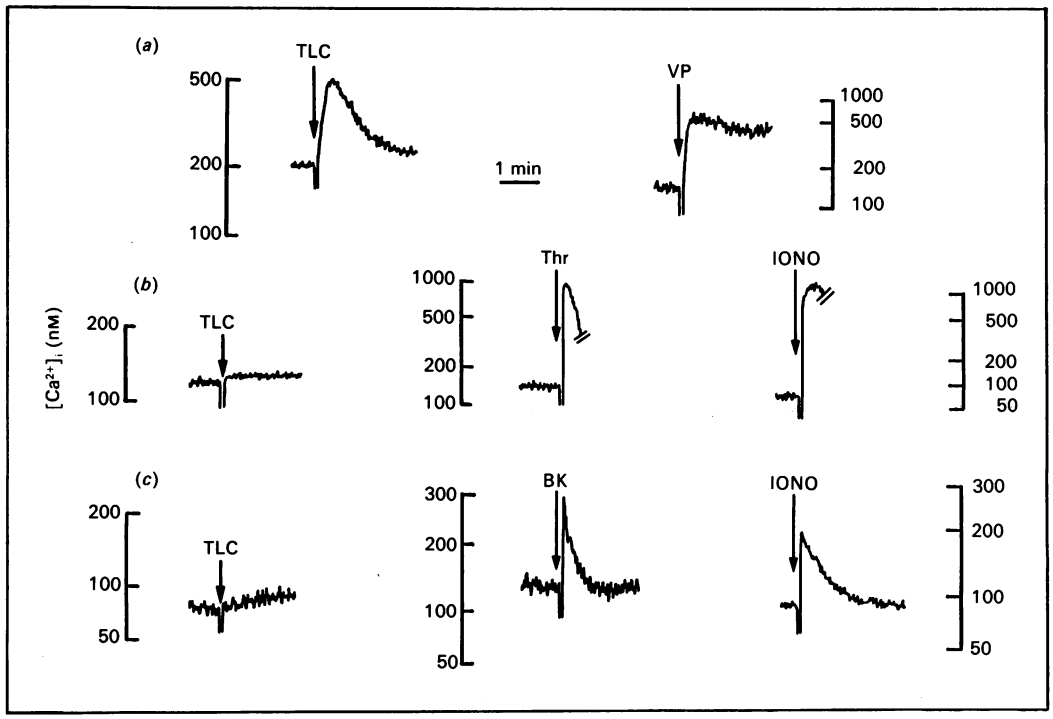

Fig. 1. Lack of effect of taurolithocholate on $[Ca^{2+}]_i$ of human platelets and neuroblastoma NG108-15 cell line

Cells were loaded with quin2 (*a*, rat hepatocytes) or fura2 (*b*, human platelets; *c*, neuroblastoma cells), and $[Ca^{2+}]_i$ was measured as described in the Materials and methods section. All the media contained Ca^{2+}, except when ionomycin was used. In these experiments, the presence of ionomycin-sensitive Ca^{2+} internal stores was checked in low-Ca^{2+} media. The chelator was added at the concentration required to lower external free Ca^{2+} to 200 nM (see the Materials and methods section). Maximal concentrations of agents were used: 100 μM-taurolithocholate (TLC), 10 nM-vasopressin (VP), 0.2 unit of thrombin/ml (Thr), 10 μM-bradykinin (BK) and 5 μM-ionomycin (IONO). Addition of equivalent volumes of the agent solvents had no effect (2.5 μl of dimethyl sulphoxide for the bile acid, or water for thrombin and bradykinin, per ml of cell suspensions). In (*b*), the traces were interrupted because the possible formation of platelet aggregates might quench the fluorescence signal of fura2.

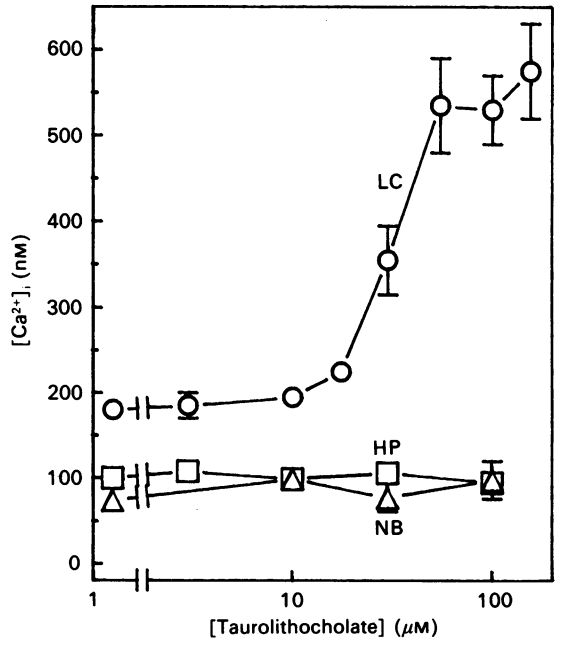

Fig. 2. Dose–response curves for the action of taurolithocholate on $[Ca^{2+}]_i$ in liver cells (○, LC), human platelets (□, HP) and neuroblastoma NG108-15 cells (△, NB)

$[Ca^{2+}]_i$ was measured as described in the Materials and methods section and in the legend to Fig. 1. The results are means ± S.E.M. from 8 (LC), 3 (HP) or 7 (NB) individual experiments. When not indicated, S.E.M. values were included within points.

valuable estimate of the kinetic properties of the Ca^{2+} release from the internal stores. Its identity suggests a common mechanism of action of taurolithocholate in the three cells.

The dose–response curves for the actions of the messenger and bile acid in human platelets, neuroblastoma cells and hepatocytes are shown in Fig. 4. As previously reported, permeabilized liver cells (Burgess *et al.*, 1984; Joseph *et al.*, 1984; Combettes *et al.*, 1989) and human platelets (Rink & Sage, 1990) displayed an $InsP_3$-dependent Ca^{2+} release. The EC_{50} values were 0.46 μM and 1.26 μM respectively. To our knowledge, no similar effect of the messenger has been reported in neuroblastoma NG108-15 cells. In our hands, these cells exhibited a rather high affinity for $InsP_3$, since the EC_{50} was 0.17 μM. This value is, however, in keeping with that determined in the parent neuroblastoma N1E-115 cell line (Chueh *et al.*, 1987). Fig. 4 also shows that half-maximal releases were observed with 34 μM-taurolithocholate in human platelets and with 15 μM in neuroblastoma cells, values that did not differ markedly from the EC_{50} displayed by liver cells (21 μM). These results show that, although ineffective in eliciting any $[Ca^{2+}]_i$ rise in intact cells, taurolithocholate is able to induce a marked release of Ca^{2+} in cells devoid of plasma membranes. This supports the view that neuroblastoma cells and human platelets do not possess the specific plasma-membrane carrier required to concentrate bile acids.

Uptake of [^{14}C]taurolithocholate

It can be predicted from the results reported above that intact human platelets and neuroblastoma cells should exhibit no significant uptake of taurolithocholate, whereas liver cells should markedly accumulate the molecule. This was examined by

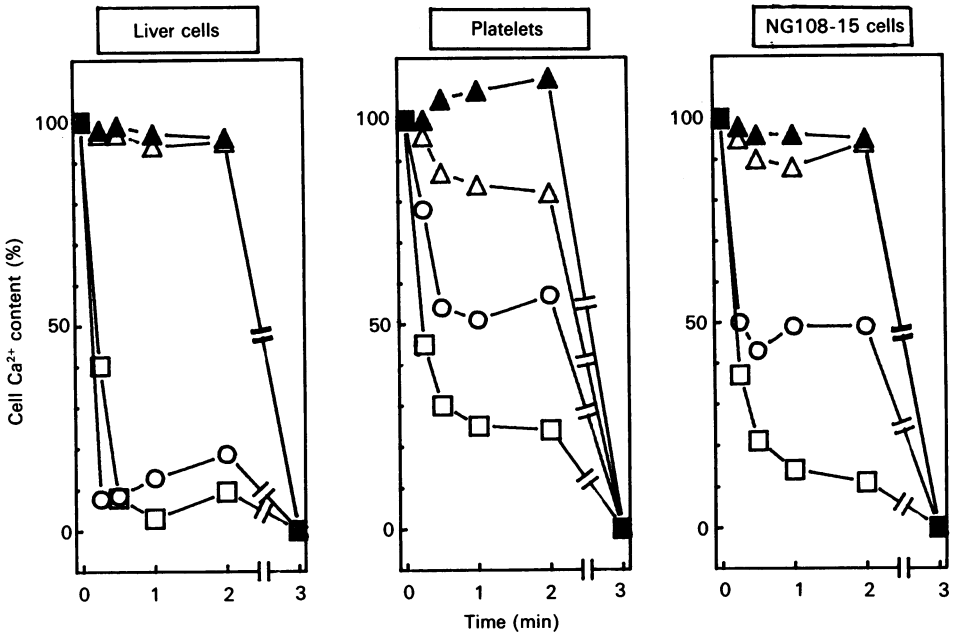

Fig. 3. Time-courses of Ca^{2+} releases induced by taurolithocholate or $InsP_3$ from permeabilized liver cells, human platelets and NG108-15 cells

Cells were permeabilized with saponin and allowed to take up Ca^{2+} to equilibrium as described in the Materials and methods section. The initial cell Ca^{2+} contents (100%) represent the percentage of the Ca^{2+} sequestered into internal pools and mobilized by 5 μM-ionomycin. They amounted to 0.83 ± 0.02 nmol/mg of protein ($n = 5$) for liver cells, 9.2 ± 1.6 nmol/mg ($n = 4$) for platelets and 5.9 ± 0.45 nmol/mg ($n = 6$) for neuroblastoma NG108-15 cells. The different agents or the equivalent volumes of their solvents were added at zero time: ▲, controls; △, 2.5 μl of dimethyl sulphoxide/ml of cell suspension; □, 100 μM-taurolithocholate; ○, 10 μM-$InsP_3$. Ionomycin was added at 2 min to deplete the Ca^{2+} remaining in the pools. The data are the means of 4–6 experiments. In contrast with liver cells and neuroblastoma cells, permeabilized human platelets appeared to be sensitive to dimethyl sulphoxide.

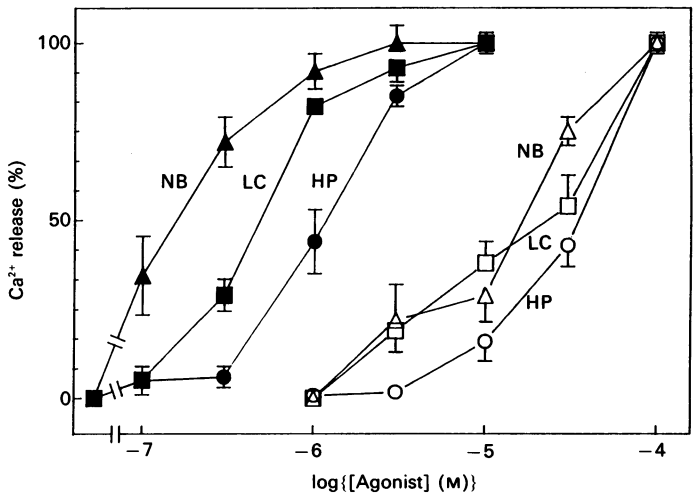

Fig. 4. Dose–response curves for the action of taurolithocholate and $InsP_3$ on Ca^{2+} release from saponin-treated cells

Liver cells (■, □; LC), human platelets (●, ○, HP) and neuronal NG108-15 cell line (▲, △; NB) were permeabilized with saponin and loaded with Ca^{2+} as described in the legend to Fig. 3 and in the Materials and methods section. Ca^{2+} releases are expressed as percentages of the maximal effect induced by each agent, as estimated from the data shown in Fig. 3. The data are means ± S.E.M. from 4–6 experiments. ▲, ■, ●, $InsP_3$; △, □, ○, taurolithocholate.

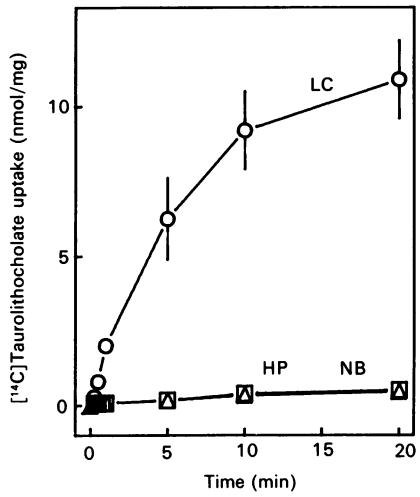

Fig. 5. Representative examples of time-courses of [^{14}C]taurolithocholate uptakes by liver cells (○, LC), human platelets (□, HP) and neuroblastoma NG108-15 cells (△, NB)

Cells were suspended in their standard media containing 100 μM-[^{14}C]taurolithocholate. At the times indicated, samples of cell suspensions were transferred into tubes containing a washing medium and centrifuged immediately as described in the Materials and methods section. Maximal uptakes observed at other [^{14}C]taurolithocholate concentrations are reported in Table 1. The results are means ± S.E.M. for 3–6 experiments.

comparing the uptake of taurolithocholate (unidirectional influx and accumulation) in the three different cells. The characteristics of taurolithocholate uptake by liver cells have been established recently (Combettes *et al.*, 1990). This study has shown that the transport is half-maximal at 25 μM-taurolithocholate and is saturated at 100 μM, in keeping with half-maximal and maximal effectiveness of the bile acid in eliciting $[Ca^{2+}]_i$ rise in intact liver cells (Figs. 1 and 2). In the present experiments, the three cells were incubated with increasing concentrations of [^{14}C]taurolithocholate from 3 to 100 μM. Influx was estimated from the initial rate of uptake. The time-courses of intracellular uptakes of [^{14}C]taurolithocholate (100 μM) are illustrated in Fig. 5. Con-

sistent with previous results (Combettes *et al.*, 1990), the uptake by liver cells was linear during the initial 2 min. Kinetic analysis between 15 and 120 s showed that the influx amounted to 2 nmol/min per mg of protein (Table 1). Isotopic equilibrium was reached at 20 min, a period of time during which liver cells accumulated 10.9 nmol of taurolithocholate/mg of cell protein (Table 1). As may be expected from the above data, there was no detectable taurolithocholate uptake in human platelets and neuroblastoma cells (Fig. 5). Only on prolonged incubation (20 min) with maximal concentration of taurolithocholate did cells reveal very small but reproducible accumulations of the bile acid, i.e. 0.4 and 0.5 nmol/mg of protein. This was also observed at the other concentrations of taurolithocholate used in these experiments. When changing the concentration, both influx and uptake were subsequently altered (Table 1). From these data, taurolithocholate influx could be estimated as 17 pmol/min per mg of protein in human platelets and neuroblastoma cells. This value is 100-fold lower than taurolithocholate influx observed in liver cells. The dose–response curve for influx in liver cells shows that half-maximal activity occurred at 25 μM-taurolithocholate (Fig. 6). Such data demonstrate that the lack of taurolithocholate-mediated $[Ca^{2+}]_i$ rise observed in intact neuroblastoma cells and human platelets results from a primary lack of a bile-acid-transporting system in their plasma membranes.

Table 1. Influx and maximal uptake of [^{14}C]taurolithocholate at different concentrations of the bile acid observed in rat liver cells, human platelets and neuroblastoma NG108-15 cell line

Cells were suspended in their standard media, then uptake was initiated by addition of 3–100 μM-[^{14}C]taurolithocholate and measured as indicated in the legend to Fig. 5 and the Materials and methods section. The influx was calculated from the initial rate of accumulation (2 min) and maximal uptake at 20 min. Note that influx and uptake are expressed in nmol in rat liver cells and in pmol in human platelets and neuroblastoma NG108-15 cells. The results are means ± S.E.M. for n experiments.

Cells	External taurolithocholate concn. (μM)	Influx (nmol/min per mg)	Maximal uptake (nmol/mg)	n
Rat liver cells	3	0.32 ± 0.05	0.67 ± 0.04	6
	10	0.70 ± 0.14	2.04 ± 0.76	6
	30	1.38 ± 0.14	6.57 ± 0.31	6
	100	2.00 ± 0.22	10.9 ± 1.3	6
		Influx (pmol/min per mg)	**Maximal uptake (pmol/mg)**	
Human platelets	3	0.06 ± 0.06	0.17 ± 0.09	3
	10	0.35 ± 0.19	5.92 ± 2.1	3
	30	2.84 ± 1.60	33.3 ± 16.8	3
	100	17.3 ± 3.2	500 ± 29	3
Neuroblastoma NG108-15 cells	3	0.63 ± 0.10	11.6 ± 2.2	4
	10	2.30 ± 0.22	39.3 ± 2.2	4
	30	9.8 ± 1.0	190 ± 24	5
	100	17.4 ± 5.9	435 ± 77	5

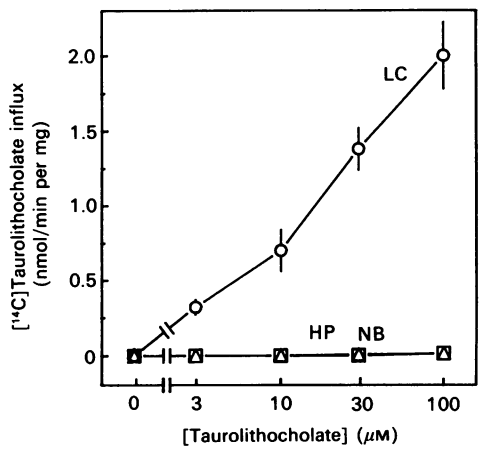

Fig. 6. Dependence of [^{14}C]taurolithocholate influx on the concentration of the bile acid measured in liver cells (○, LC), human platelets (□, HP) and neuroblastoma NG108-15 cells (△, NB)

The influx was calculated from the initial rate of uptake (2 min) under conditions described in the legend to Fig. 5 and in the Materials and methods section. The absolute values for human platelets and neuroblastoma NG108-15 cells are shown in Table 1. The results are means ± S.E.M. for 3–6 experiments.

DISCUSSION

The present study was undertaken to assess whether the Ca^{2+}-permeabilizing effect of monohydroxy bile acids is restricted to the liver, since a wide variety of cell types can be in a durable contact with these natural circulating molecules. The experiments have attempted to identify and characterize the response of three different cells to the same treatment with taurolithocholate. The major interest in utilizing human platelets (Rink & Sage, 1990) and the neuronal NG108-15 cell line (Hamprecht, 1977; Nirenberg *et al.*, 1983) resides in the fact that they are genetically and embryologically unrelated to hepatocytes. Since neural tissues *in situ* are not accessible to direct measurement of cytosolic Ca^{2+} with fluorescent markers, the well-defined neuroblastoma cell line NG108-15 is an excellent tool for elucidation of the mechanisms by which the agonists may exert their effects. Indeed, these cells show a number of morphological, biochemical and physiological properties expressed by functionally differentiated neuronal cells (Hamprecht, 1977; Nirenberg *et al.*, 1983). In addition, both human platelets and neuroblastoma NG108-15 cells possess respectively the thrombin and bradykinin receptors coupled to polyphosphoinositide hydrolysis and the $InsP_3$-dependent Ca^{2+} pool (Agranoff *et al.*, 1983; Reiser & Hamprecht, 1985; Higashida *et al.*, 1986; Pollock & Rink, 1986; Jackson *et al.*, 1987; Rink & Sage, 1990).

In these experiments, we compared in the three cell types the effects of taurolithocholate on the uptake of the bile acid, on $[Ca^{2+}]_i$ in intact cells and on Ca^{2+} release from permeabilized cells. Whereas their $[Ca^{2+}]_i$ was increased by their relevant agonists, intact human platelets and neuroblastoma NG108-15 cell line were not affected by the bile acid at concentrations that were highly effective in the rat hepatocyte. The finding that the influx of taurolithocholate was 100-fold greater in liver cells supports the view that the other cells do not express this carrier. It should be noted that the discrepancy between the effects of taurolithocholate in neuroblastoma NG108-15 cells and liver cells may not be explained by the fact that neuroblastoma NG108-15 cells are transformed hybrid cells (Hamprecht, 1977; Nirenberg *et al.*, 1983) and that they lack essential components that are targets for the taurolithocholate effect, since freshly prepared human platelets appear to be similarly resistant. Therefore the lack of acute effect of taurolithocholate in intact cells is a characteristic of the deficiency of the taurolithocholate–carrier complex. This is consistent with the fact that, to our knowledge, a specific transport system for bile acids in mammalian cells has only been identified in hepatocytes and enterocytes (Carey & Cahalane, 1988). Thus the potential ability of plasma taurolithocholate in altering the $[Ca^{2+}]_i$ *in vivo* is

unlikely to be a widespread phenomenon, and should be restricted to a limited number of particular organs.

In contrast, a number of results have established the kinetic and molecular properties of the carrier in the liver, at least for the one involved in the transport of the most common trihydroxy bile acids, such as taurocholate and cholate. Unlike monohydroxy bile acids, these stimulate bile secretion (Boyer, 1986; Erlinger, 1987). The uptake and accumulation by the liver of these negatively charged molecules against unfavourable electrical and chemical gradients is predominantly mediated by the secondary active-transport process driven by the out-to-in Na^+ gradient, which exhibits a K_m for taurocholate uptake in the range of 30 μM (Coleman, 1987; Frimmer & Ziegler, 1988; Meier, 1989). A protein of 49 kDa has been identified and shown to be involved in the Na^+-dependent bile-acid uptake by recent monoclonal-antibody studies (Ananthanarayanan *et al.*, 1988). We have shown here and in a previous study (Combettes *et al.*, 1990) that the liver cells also actively accumulate taurolithocholate, with an EC_{50} of 24 μM. However, very few data have been reported on the transport system responsible for their entry into the cell. Particularly, it is not known whether taurolithocholate utilizes the same carrier as that used by the trihydroxy bile acids. Taurolithocholate has been reported to inhibit taurocholate uptake (Schwenk *et al.*, 1977), and preliminary experiments undertaken in our laboratory show that approx. 60% of taurolithocholate influx in liver is Na^+-dependent (B. Berthon, unpublished work). Taken together, these data suggest a common molecular mechanism, dependent on Na^+, for uptake of both mono- and tri-hydroxy conjugated bile acids in the liver.

Another important part of our results has been obtained from the release of Ca^{2+} from permeabilized cells. This experimental model has been largely used to assess whether the normally non-permeant intracellular messengers can mimic the effects of hormonal stimuli. In liver cells, human platelets and in the parent neuronal cell line N1E-115, reports have clearly identified the ER, or specialized parts of it, as the physiologically relevant internal Ca^{2+} pool, sensitive to $InsP_3$ (Gill & Chueh, 1985; Burgess *et al.*, 1984; Joseph *et al.*, 1984; Chueh *et al.*, 1987; Combettes *et al.*, 1988*a*; Yoshida *et al.*, 1988; Rink & Sage, 1990). Unexpectedly, we have found that the two tested cells that are insensitive to extracellular taurolithocholate possessed the intracellular target for monohydroxy bile acids, and that removing the plasma membrane unmasked the releasing ability of the molecules. Thus, at variance with the data observed in intact cells, little difference could be found between the three types of permeabilized cells. Taurolithocholate-mediated Ca^{2+} releases in platelets and neuroblastoma cells share apparently similar kinetic properties with the taurolithocholate system in the liver. A major fraction of the Ca^{2+} sequestered was releasable, with EC_{50} of 15–34 μM. The rate constants of the effects (approx. 4–5 min^{-1}) were also comparable, suggesting a common mechanism of permeabilization. Though relatively fast, this effect is, however, much slower than that produced by $InsP_3$, which opens Ca^{2+} channels within 10 ms and releases Ca^{2+} within 100–250 ms, as estimated by a stopped-flow method or by photolytic release of caged $InsP_3$ (Champeil *et al.*, 1989; Ogden *et al.*, 1990; Meyer *et al.*, 1990). Other studies are required to analyse the molecular mechanism by which taurolithocholate affects the ER of mammalian cells. From previous data reported in isolated rat liver cells (Combettes *et al.*, 1988*a*; Anwer *et al.*, 1988), the effect of taurolithocholate appears to be transient (5 min) in intact cells, whereas it is sustained in permeabilized cells or in a microsomal fraction from rat liver (Combettes *et al.*, 1989). It has been proposed that this difference could result from a greater binding to internal protein in intact cells than in permeabilized cells or in subcellular fractions of the ER. It has also been shown that the permeabilizing effect of the monohydroxy bile acids, including taurolithocholate, is rather selective for the membrane of the ER. No effect is observed on the other natural membranes, such as cell plasma membrane and mitochondrial membrane, or on the permeability of artificial membranes, at least in the range of Ca^{2+} concentrations used here (3–100 μM). Its effect is not abolished by the Ca–Mg-ATPase inhibitor vanadate. Thus this action seems to differ from the detergent-like effect of monohydroxy bile acids on Ca^{2+} movements reported by others at higher concentrations (Maenz & Forsyth, 1984; Schölmerich *et al.*, 1984; Oelberg *et al.*, 1986, 1987; Anwer *et al.*, 1988).

In summary, our results have characterized the spectrum of the target tissues that are potentially sensitive to taurolithocholate. In intact cells, taurolithocholate has no effect on $[Ca^{2+}]_i$ of human platelets and of the neuroblastoma cells NG108-15, whereas it markedly alters that of liver cells. This results from a primary lack of the carrier system responsible for taurolithocholate uptake in the liver. Paradoxically, the action of taurolithocholate is observed in the cells in which the plasma membrane has been permeabilized. These results have implications toward not only regulation of Ca^{2+} in response to circulating bile acids, but also the use of the family of monohydroxy bile acids as tools to deplete internal pools specifically, including the $InsP_3$-sensitive pool, provided that the plasma membrane has been previously permeabilized.

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