Effect of Bile Acids on Calcium Efflux from Isolated Rat Hepatocytes and Perfused Rat Livers (42900)

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Abstract. **The changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of hepatocytes induced by certain bile acids are biphasic: an initial increase is followed by a more gradual decrease. This latter decline in $[Ca^{2+}]_i$ may be due to an efflux of Ca^{2+} across the plasma membrane. This hypothesis was tested by studying the effect of different bile acids on the efflux of ^{45}Ca from preloaded rat hepatocytes and isolated perfused rat livers. The following bile acids were studied: cholic (C), ursodeoxycholic (UDC), chenodeoxycholic (CDC), and deoxycholic (DC) acids; their taurine (T) conjugates (TC, TUDC, TCDC, and TDC); and the taurine, sulfate (S), and glucuronide (Glu) derivatives of lithocholic acid (TLC, LS, TLS, and LGlu, respectively). At 0.3 m*M*, all bile acids except C, TC, TCDC, UDC, and TUDC significantly increased ^{45}Ca efflux from preloaded hepatocytes without affecting cell viability. Dose-response studies revealed that the minimum effective concentration needed to induce ^{45}Ca efflux was 0.06 m*M* for LS, 0.8 m*M* for TCDC, and 10 m*M* for TC. Efflux of ^{86}Rb from preloaded hepatocytes was not significantly altered by 0.1 m*M* LS, indicating relative specificity for calcium. TDC and DC, but not TC, increased ^{45}Ca efflux from preloaded perfused rat livers. These results showed that bile acids known to increase $[Ca^{2+}]_i$ (CDC, DC, TDC, and TLC) also increased ^{45}Ca efflux from hepatocytes and perfused livers and that efflux was also stimulated by LS, TLS, and LGlu. The extent of this efflux was related to the hydrophobicity of the steroid nucleus of the bile acid. It is speculated that bile acid-induced increases in $[Ca^{2+}]_i$ activate the plasma membrane Ca^{2+} pump resulting in increased Ca^{2+} efflux.**

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Cytosolic calcium activity, $[Ca^{2+}]_i$, of hepatocytes is maintained at a low resting level (Ca_i 0.2 μM) against an extracellular concentration of 1.3 m*M* (1, 2). This is accomplished by sequestration into mitochondria, by uptake into the endoplasmic reticulum, and by an energy-dependent Ca^{2+} extrusion mechanism, the Ca^{2+} pump, at the plasma membrane (1, 2). It is now recognized that a number of cellular processes, such as muscle contraction, stimulus-secretion coupling, hormone action, and cell death, are mediated and/or regulated via changes in $[Ca^{2+}]_i$ (3–5). Recent studies indicate that bile acids increase calcium transport into red blood cells (6), colonic epithelial cells (7), pig jejunal brush border membranes (8, 9), and liposomes (10) and increase $[Ca^{2+}]_i$ in kidney cell lines (11) and hepatocytes (12, 13). In addition, bile acids act as Ca^{2+} ionophores in model membranes (14, 15). The ionophoric properties of bile acids are related to the hydrophobicity of the steroid nucleus. Thus, the ability of bile acids to raise $[Ca^{2+}]_i$ increases with increasing hydrophobicity.

Bile acid-induced increases in $[Ca^{2+}]_i$ may involve both uptake from extracellular medium and release from intracellular stores. In isolated hepatocytes, changes in $[Ca^{2+}]_i$ in response to bile acids are biphasic: an initial rapid increase in $[Ca^{2+}]_i$ is followed by a gradual return to baseline (12, 13). Although the rapid initial increase in $[Ca^{2+}]_i$ may be due to the ionophoric activity of bile acids, the latter phase of gradual decline in $[Ca^{2+}]_i$ may be due to intracellular sequestration and/or efflux across the plasma membrane. The possibility that the observed decrease in $[Ca^{2+}]_i$ is due to efflux was investigated in this study by determining the effect of bile acids on ^{45}Ca efflux from isolated rat hepatocytes and in isolated perfused rat livers preloaded with ^{45}Ca. The results showed that bile acids known to

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increase $[Ca^{2+}]_i$ increase ^{45}Ca efflux and that the extent of this efflux is related to the hydrophobicity of the bile acid nucleus.

Materials and Methods

Preparation of Isolated Hepatocytes. Isolated hepatocytes were prepared from livers of male Sprague-Dawley rats (250–350 g) using the perfusion protocol described by Blitzer *et al.* (16). Following digestion, the liver was removed and the capsule peeled back. The liver was cut into pieces, placed in 50 ml of fresh digestion buffer, and incubated for 10 min at 37°C, with shaking, under an atmosphere of 95% O_2-5% CO_2. This crude suspension was then filtered once through cheesecloth and once through nylon mesh (62-μm pore size; Tetko, Elmsford, NY) and was centrifuged at 50*g* for 1.5 min; the supernatant was aspirated off and the cells were washed three times with wash buffer (modified Hanks' buffer containing 0.25% gelatin). At this point, hepatocyte viability and cell number were assessed by adding 1 volume of the cell suspension to 4 volumes of 0.4% trypan blue in saline.

Efflux of ^{45}Ca or ^{86}Rb from Isolated Hepatocytes. After washing the cells twice with incubation buffer (Hanks' buffer without phosphate, containing 1 m*M* $CaCl_2$, 15 m*M* Hepes, and 0.25% gelatin), viability and cell number were again determined. An aliquot of the hepatocyte suspension (about 2×10^6 cells/ml) was incubated with ^{45}Ca (1 μCi/ml) or ^{86}Rb (0.5 μCi/ml) for 1 hr at 37°C, with shaking, to load the cells with ^{45}Ca or ^{86}Rb. At the end of the loading period, successive aliquots of cells (2.0 ml) were quickly washed to remove extracellular ^{45}Ca or ^{86}Rb and resuspended in the same buffer without ^{45}Ca or ^{86}Rb. Two 150-μl aliquots were taken as 0 time samples. One milliliter of the remaining suspension was transferred to a vial, an appropriate volume of the test agent was added and the cells were incubated at 37°C for 5 min. Aliquots of the suspension were withdrawn at 1 and 5 min after addition of the test agent.

In a separate series of experiments, hepatocytes were incubated with ^{45}Ca (0.5 μCi/ml) for 60 min followed by addition of TC, TDC, DC (final concentration, 0.3 m*M*), or buffer (control) without washing out extracellular ^{45}Ca. Samples were taken every 20 min before addition of bile acid and at 5, 10, 15, 20, and 30 min after addition of bile acid. Effect of bile acid on cellular content of ^{45}Ca was determined and expressed as percentage of the 60-min value.

At the time of sampling, aliquots of the cell suspension were placed in microtubes containing silicone oil (density = 1.050; Aldrich Chemical Co., Milwaukee, WI) layered over 3 *M* KOH and cells were separated from incubation medium by rapid centrifugation for 15 sec in a Beckman Microfuge (model B; Beckman Instruments, Palo Alto, CA). An aliquot of the supernatant was taken for lactate dehydrogenase (LDH) determination; the tip of the tube containing the cell pellet was cut just above the oil-KOH interface and the protein was allowed to digest overnight. The pellet digest was diluted in water and aliquots were taken for determination of cell protein and radioactivity. Isotope remaining in the cell pellet at the specified time of sampling was determined and results are expressed as percentage of the control value.

Efflux of ^{45}Ca from Perfused Rat Livers. Livers from male rats were surgically isolated as previously described (17) and were perfused under a hydrostatic pressure of 15 cm and at a constant flow rate of 40 ml/min. The composition of the standard perfusate was 120 m*M* NaCl, 4.7 m*M* KCl, 26 m*M* $NaHCO_3$, 1.2 m*M* KH_2PO_4, 1.3 m*M* $CaCl_2$, 0.6 m*M* $MgSO_4$, and 5.5 m*M* glucose. The perfusate was gassed with 95% O_2/5% CO_2, and the pH was maintained at 7.4.

Following a 20-min stabilization period, each liver was perfused with a recirculating perfusate containing ^{45}Ca (100 μCi/100 ml) for 60 min to load the liver with ^{45}Ca. This was followed by nonrecirculating perfusion with ^{45}Ca-free perfusate for 20 min. This final nonrecirculating perfusion was divided into three periods: an initial 5-min perfusion with standard perfusate, a second 5-min perfusion with either standard perfusate alone (control) or perfusate containing 0.3 m*M* bile acid, and a final 10-min perfusion with standard perfusate. Perfusate leaving the liver was sampled at short intervals to define the characteristics of ^{45}Ca efflux.

Analytical Techniques. Hepatocyte ^{45}Ca or ^{86}Rb content was determined by scintillation counting in Liquiscint cocktail (National Diagnostics, Somerville, NJ) using a Tracor Mark III model 6882 liquid scintillation spectrometer (TM Analytic, Elk Grove Village, IL). Protein was determined according to the method of Lowry *et al.* (18) and LDH was measured using an LDH diagnostic kit (#340-UV; Sigma Chemical Co., St. Louis, MO). Total hepatocyte LDH was determined in the supernatant of cell suspensions that had been lysed with 0.1% Triton X-100 (Sigma). Extracellular water entrained by hepatocytes during centrifugation was determined by incubating cells with ^3H-polyethylene glycol-4000 (New England Nuclear, Boston, MA) and assaying cell pellets for ^3H content. Oxygen consumption was measured with YSI model 53 oxygen monitor and probe (Yellow Springs Instrument Co., Yellow Springs, OH).

The sodium salts of the following bile acids were used: cholic acid (C), chenodeoxycholic acid (CDC), deoxycholic acid (DC), ursodeoxycholic acid (UDC); and their taurine conjugates (TC, TCDC, TDC, TUDC), the taurine, 3-sulfate and 3-glucuronide conjugates of lithocholic acid (TLC, LS, LGlu), taurolithocholic acid-3-sulfate (TLS), and the 3-glucuronide of 3β-hydroxy-5-cholenoic acid (ΔGlu). All bile acids were obtained from CalBiochem (LaJolla, CA) except for the glucuronide conjugates which were synthesized in our

laboratory (19). Stock solutions of all bile acids except TLC and LS were prepared in water at 50–100 times the concentration desired for incubation. Stock solutions of TLC and LS were solubilized in water by heating and were kept warm until added to the incubation suspension.

Calcium-45 and rubidium-86 were obtained from ICN Pharmaceuticals (Irvine, CA) and were diluted to the desired specific activity with incubation buffer. A23187 (CalBiochem) was dissolved in dimethyl sulfoxide at concentrations of 1.0 or 0.1 m*M*. Ionomycin (CalBiochem) was solubilized in 95% ethanol at a stock concentration of 0.1 m*M*.

Results

Viability of Isolated Rat Hepatocytes. By light microscopy, suspensions prepared by the procedure outlined above were 99% parenchymal cells which were rounded with refractory edges. Trypan blue was excluded by 90–95% of the cells and this value correlated well with LDH retention by the cells and with protein recovery from cell pellets after centrifugation through silicone oil (nonviable cells are not dense enough to sediment through the oil). Oxygen consumption by freshly prepared cells was 2.11 ± 0.21 nmol/min/mg protein and was stimulated less than 20% by 1 m*M* succinate and more than 50% by 1 μm of carbonyl cyanide 3-chlorophenylhydrazone. As judged by LDH levels in the supernatants (Table I) and by cell counts done at the beginning and end of each experiment, viability decreased a maximum of 10% over the course of the incubations (approximately 45 min).

Refrigeration of the stock suspension for up to 4 hr between experiments resulted in a 10–30% decrease in cell number. This was determined after washing twice to remove nonviable cells. The remaining cells had a normal appearance and greater than 90% trypan blue exclusion. Results of studies using refrigerated cells were not significantly different from those using freshly prepared cells.

As measured by ^{3}H-polyethylene glycol-4000 (M_r = 4000), extracellular water in cell pellets was 1–2 μl/mg protein. The correction for adherent extracellular fluid represented less than 1% of the total cellular radioactivity.

Effects of Bile Acids on ^{45}Ca Efflux from Hepatocytes. The effect of bile acid on cellular content of ^{45}Ca was not significantly different from control at 1 min. Thus, results obtained at 5 min are reported. Under control conditions (no bile acid added), ^{45}Ca content of hepatocytes decreased to 63% of the initial value after 5-min incubation of preloaded cells in ^{45}Ca-free buffer. Comparison of this value to those obtained in the presence of different bile acids (0.3 m*M*) gave the results shown in Figure 1. Of the free and taurine-conjugated bile acids tested, CDCA, DCA (Fig. 1A), and TLC (Fig. 1B) decreased ^{45}Ca content of hepatocytes by 35–40% as compared with controls; TDC also

Table I. Effect of 0.3 m*M* Bile Acid on LDH Release to Medium[a]

Agent	LDH release (% of total)	Agent	LDH release (% of total)
Control	4.4 ± 0.4		
C	4.6 ± 0.8	TLC	3.5 ± 0.5
UDC	3.0 ± 0.9	LS	1.3 ± 0.3
CDC	4.8 ± 0.4	TLS	1.8 ± 0.7
DC	7.0 ± 0.9[b]	LGlu	2.5 ± 0.5
TC	2.5 ± 0.4	Δ5Glu	4.4 ± 1.0
TUDC	3.0 ± 0.6	A23187	4.9 ± 1.0
TCDC	2.3 ± 0.5	Ionomycin	7.8 ± 1.1[b]
TDC	5.8 ± 0.8		

[a] LDH was measured in the supernatant of cells pelleted after 5-min incubation (see text for details of incubation). Total hepatocyte LDH was determined on an aliquot of cells lysed (from lysis) with 0.1% Triton X-100.
[b] $P < 0.01$ vs control (paired t test).

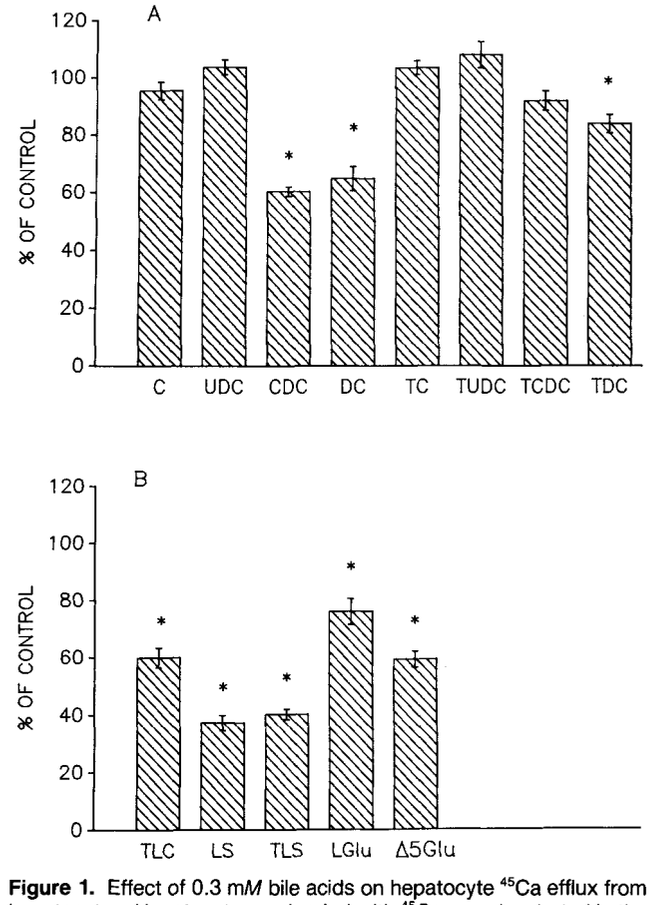

Figure 1. Effect of 0.3 m*M* bile acids on hepatocyte ^{45}Ca efflux from hepatocytes. Hepatocytes preloaded with ^{45}Ca were incubated in the presence of 0.3 m*M* bile acid (see text for incubation conditions) and the isotope remaining in the cells after 5 min was compared with that in control (no bile acid) incubations. Results are expressed as a percentage of the control value. The bars give the mean of at least four experiments from at least two different cell preparations and the brackets give the standard error. Values (original counts) are compared using the paired t test. * = $P < 0.01$ vs control. Abbreviations as in text.

reduced hepatocyte ^{45}Ca significantly but to a lesser degree. The effects of 0.3 m*M* C, UDC, TC, TCDC, and TUDC (Fig. 1A) were not significantly different from controls. On the other hand, all of the lithocholic acid derivatives and the glucuronide conjugate of 3β-hydroxy-5-cholenoic acid (Fig. 1B) significantly reduced ^{45}Ca content at 0.3 m*M*, and sulfate conjugates were more effective than glucuronides. Thus, the relative ability of various bile acids at 0.3 m*M* to increase ^{45}Ca efflux was in the following order: LS = TLS > Δ 5Glu = CDC = DC = TLC > LGLU > TDC > TCDC ≫ C, TC, UDC, TUDC. Dose-response curves for LS, TCDC, and TC (Fig. 2) showed that the minimum effective concentrations were 0.06 m*M* for LS, 0.8 m*M* for TCDC, and 10 m*M* for TC. Taken together, these results show that at 0.3 m*M*, monohydroxy bile acids are most effective in increasing Ca^{2+} efflux followed by dihydroxy (except UDC) and then trihydroxy bile acids.

In the above-mentioned studies, bile acids induced ^{45}Ca efflux from preloaded hepatocytes. Since some of these bile acids have been shown to be Ca^{2+} ionophores, it is possible that the enhanced ^{45}Ca efflux is due to isotope exchange, i.e., mass of Ca^{2+} going in and ^{45}Ca going out. To rule out this possibility, the effect of bile acid on ^{45}Ca movement was studied in the presence of extracellular ^{45}Ca. Under these experimental conditions, TDC and DC, but not TC, decreased cellular content of ^{45}Ca (Fig. 3), indicating that efflux of ^{45}Ca induced by bile acids represents mass movement and is not due to isotope exchange alone.

Effect of Calcium Ionophores on ^{45}Ca Efflux from Hepatocytes. The ability of bile acids to increase ^{45}Ca efflux may be related to their Ca^{2+} ionophoric activity. In that case, Ca^{2+} ionophores, such as A23187 and ionomycin, should also increase ^{45}Ca efflux. A23187 (10 μ*M*) and ionomycin, (1μ*M*) decreased ^{45}Ca content of hepatocytes to 28.5 ± 1.1 and 24.1 ± 0.6% of control, respectively, in 5 min (n = 8). Even after only 1-min incubation, hepatocyte ^{45}Ca content was half that of the controls. Thus, Ca^{2+} ionophores also increased ^{45}Ca efflux from hepatocytes.

Calcium Specificity of Bile Acid-Induced Efflux. To examine whether the effect of bile acid on ^{45}Ca efflux was due to a general increase in membrane permeability to ions, the effect of LS (0.1 m*M*) on ^{86}Rb efflux from preloaded hepatocytes was investigated. Although LS decreased ^{45}Ca content of hepatocytes to 39 ± 3.2% of control (n = 4) in 5 min, it failed to significantly decrease ^{86}Rb content (95.3 ± 1.6% of control after 5 min; n = 5). Thus, efflux is not a function

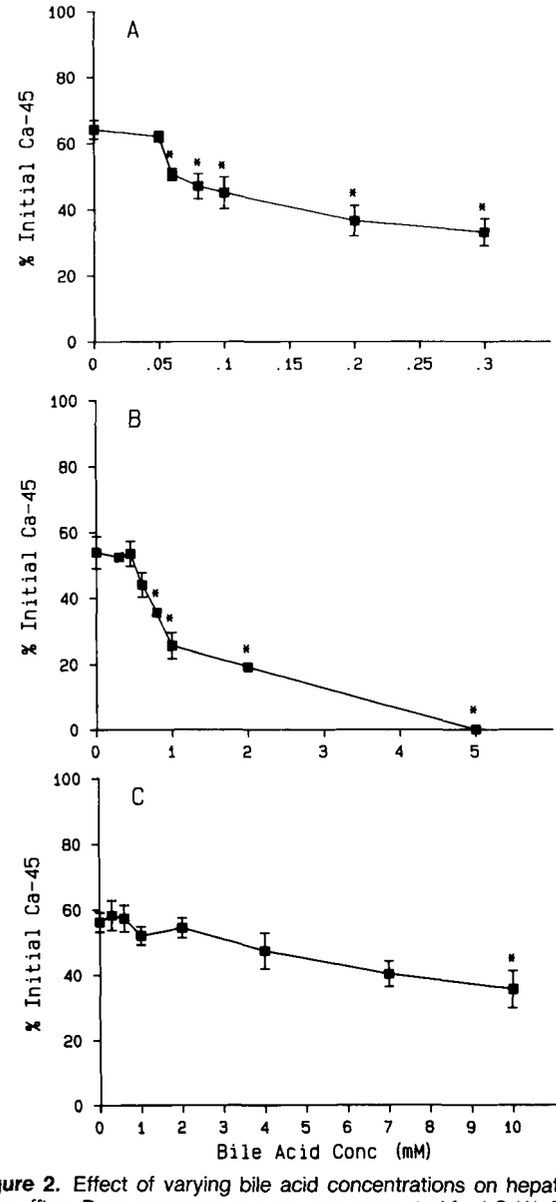

Figure 2. Effect of varying bile acid concentrations on hepatocyte ^{45}Ca efflux. Dose-response curves were generated for LS (A), TCDC (B), and TC (C). Details as described in Figure 1, except that cells were incubated with increasing concentrations of bile acids and data are presented as a line graph. Values (mean ± SE) are compared using the paired t test. * = $P < 0.01$ vs control (0 m*M* bile acid).

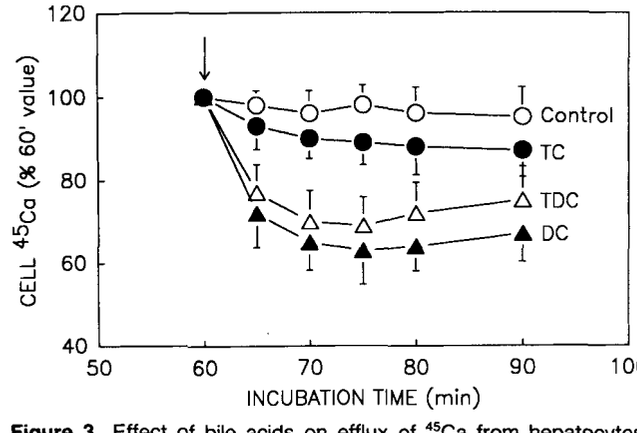

Figure 3. Effect of bile acids on efflux of ^{45}Ca from hepatocytes. Following 60-min incubation of hepatocytes in the presence of ^{45}Ca, buffer (control), TC (1 m*M*), DC (0.3 m*M*), or TDC (0.3 m*M*) was added. Cell-associated radioactivity was determined at indicated times and expressed as percentage of the 60-min value. Data represent mean ± SEM (n = 6).

of increased membrane permeability and is at least partially specific for Ca^{2+}.

Efflux of ^{45}Ca from Perfused Rat Liver. To determine whether bile acids also induced efflux of Ca^{2+} in intact liver, the effect of DC, TDC, and TC on ^{45}Ca efflux was studied in isolated perfused rat livers. Following preloading of livers with ^{45}Ca, perfusate ^{45}Ca concentrations declined with time during perfusion with standard perfusate (control, ^{45}Ca-free) (Fig. 4). The appearance of ^{45}Ca during TC infusion was similar to that seen in control perfusions. However, perfusate ^{45}Ca increased (relative to control) during infusion of DC and TDC (Fig. 4). DC was more effective than TDC in increasing perfusate ^{45}Ca.

Discussion

Previous studies have shown that bile-induced changes in $[Ca^{2+}]_i$ involve a rapid initial increase followed by a gradual decline to baseline values (12, 13). In this study, we have examined the effect of a series of bile acids on ^{45}Ca-loaded hepatocytes to determine whether the observed decline in $[Ca^{2+}]_i$ is due to ^{45}Ca efflux. The results indicated that bile acids (CDC, DC, TDC, LC, and TLC) previously shown to increase $[Ca^{2+}]_i$ in hepatocytes (12, 13), liposomes (14), and kidney cell lines (13) also increased ^{45}Ca efflux from isolated rat hepatocytes (Fig. 1). This increased efflux was not due to gross cell damage (Table I) and appeared to be at least partially specific for calcium. The results also showed that bile acids, such as TC and C, which do not increase $[Ca^{2+}]_i$, did not affect ^{45}Ca efflux. In addition, the effects of DC, TDC, and TC on ^{45}Ca efflux from isolated perfused rat liver were examined. As in the isolated hepatocyte preparation, DC and TDC, but not TC, significantly increased efflux of ^{45}Ca from the perfused liver into the perfusate (Fig. 4). Additional studies indicate that bile acid-induced increases in ^{45}Ca efflux are not due to isotope exchange (Fig. 3). These studies show that bile acids previously shown to increase $[Ca^{2+}]_i$ also increase Ca^{2+} efflux from hepatocytes.

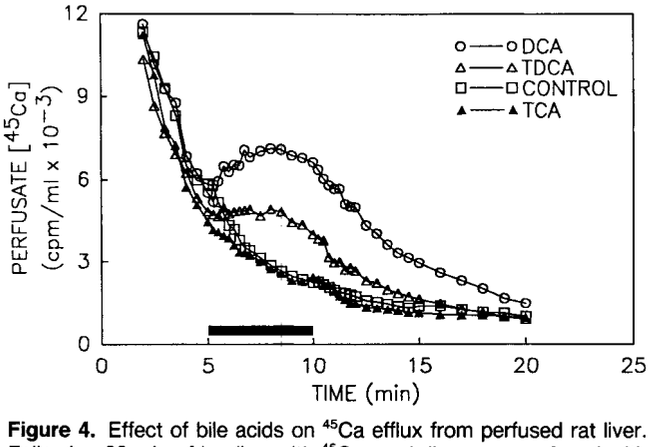

Figure 4. Effect of bile acids on ^{45}Ca efflux from perfused rat liver. Following 60 min of loading with ^{45}Ca, each liver was perfused with a nonrecirculating perfusate without ^{45}Ca for 20 min. Duration and time of perfusion with bile acid (0.3 m*M*) is indicated by the horizontal bar. The data are from one experiment representative of three separate experiments. Control, perfusate without bile acid; DCA, deoxycholate; TDCA, taurodeoxycholate; and TCA, taurocholate.

Results of our study also suggest that the ability of bile acids to increase Ca^{2+} efflux may be related to their hydrophobicity. The hydrophobicity of bile acids, with the exception of UDC, decreases with increasing hydroxylation of the steroid nucleus (20). Present studies show that monohydroxy bile acids are most effective in increasing Ca^{2+} efflux followed by dihydroxy (except UDC) and then trihydroxy bile acids. Bile acids have been shown to act as Ca^{2+} ionophores and this activity also increases with increasing hydrophobicity (10, 21). Therefore, it is likely that the ability of bile acids to increase Ca^{2+} efflux may be related to their Ca^{2+} ionophoric activity and the consequent increase in $[Ca^{2+}]_i$. This is consistent with the results that the Ca^{2+} ionophores, A23187 and ionomycin, also increase ^{45}Ca efflux. Although certain bile acids increase $[Ca^{2+}]_i$, the source of this Ca^{2+} (extracellular vs intracellular) is not clear. Results of our previous studies indicate that both extracellular and intracellularly stored Ca^{2+} may be involved (12). However, another study reported that bile acids are released Ca^{2+} from hepatic endoplasmic reticulum only, and this release does not involve inositol trisphosphate (13). This difference, however, does not exclude the possibility that bile acid-induced Ca^{2+} efflux is related to the Ca^{2+} ionophoric activity of bile acids, and hence to their ability to increase $[Ca^{2+}]_i$. Since bile acids are taken up by hepatocytes, bile acids could act as Ca^{2+} ionophores at the endoplasmic reticulum as well as at the plasma membrane resulting in an increase in $[Ca^{2+}]_i$.

The mechanism by which certain bile acids increase hepatic Ca^{2+} efflux cannot be answered from our study. However, a possible mechanism can be proposed based on our present understanding of the regulation of hepatic Ca^{2+} transport. Under the present experimental conditions, extracellular $[Ca^{2+}]$ (1 m*M*) is at least three order of magnitude higher than $[Ca^{2+}]_i$ (<1 μM). Thus, bile acid-induced Ca^{2+} efflux is occurring against an electrochemical gradient, and hence must be due to an active transport of Ca^{2+} out of the cells. Active transport of Ca^{2+} across the hepatocyte plasma membrane is believed to be primarily via the Ca^{2+} pump. The presence of a Na^+/Ca^{2+} antiport at the hepatocyte plasma membrane is equivocal (22, 23). Bile acids may directly or indirectly activate the Ca^{2+} pump. Inasmuch as certain bile acids (LC, TLC, CDC, TCDC, and TC) have been shown to inhibit Na^+,K^+-ATPase (24, 25) and the Ca^{2+}-ATPase (Ca^{2+} pump) bears a remarkable structural and mechanistic similarity to the Na^+,K^+-ATPase (26), it seems unlikely that bile acids increase Ca^{2+} efflux by directly activating the Ca^{2+} pump. Since the activity of the Ca^{2+} pump is regulated, in part, by $[Ca^{2+}]_i$, and bile acids shown to

increase $[Ca^{2+}]_i$ also increase Ca^{2+} efflux, it is more probable that bile acids stimulate the Ca^{2+} pump indirectly by increasing $[Ca^{2+}]_i$. However, indirect activation of the Ca^{2+} pump by other mechanisms cannot be ruled out.

This study shows that bile acids known to increase $[Ca^{2+}]_i$ also increase Ca^{2+} efflux from isolated hepatocytes and perfused rat livers. The ability of bile acids to increase Ca^{2+} efflux is related to their hydrophobicity. It is speculated that bile acid-induced increases in $[Ca^{2+}]_i$ activate the plasma membrane Ca^{2+} pump resulting in increased Ca^{2+} efflux.

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