Taurine-Conjugated Bile Acids Act as Ca^{2+} Ionophores†

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ABSTRACT: The ionophoretic properties of several taurine-conjugated bile acids have been investigated in two experimental systems: in a two-phase bulk partitioning system and in proteoliposomes. In the former, a bile acid/Ca^{2+} complex was extracted into the bulk organic phase and had an experimental stoichiometry of 1.75. Extraction was specific for Ca^{2+} over Mg^{2+}; Na^+ and K^+ did not compete with the extraction of Ca^{2+}. In the second system, bile acids at concentrations as low as 5–100 molecules/vesicle lowered the steady-state Ca^{2+} gradient maintained by a reconstituted sarcoplasmic reticulum Ca^{2+}–ATPase. The effect was not due to nonspecific membrane perturbation. In addition to releasing intravesicular Ca^{2+} in a transmembraneous process, bile acids caused partition of Ca^{2+}/bile acid complexes into the hydrophobic core of the bilayer. In both experimental systems, the Ca^{2+} ionophoretic activity correlated well with the concentration and the hydrophobicity of the bile acid. Taurolithocholate was most active, with a significant effect measurable at 10 μM in either system. Since bile acid concentrations equal to those used in our experiments can occur in the blood in certain liver diseases, the results support the notion that bile acids can increase the intracellular Ca^{2+} concentration bypassing the regulatory systems that maintain cellular Ca^{2+} homeostasis.

Some, but not all, amphiphilic molecules can act as ionophores for metal ions. Several conditions must be met by an agent to function in this manner. The binding of the ion must be specific, which requires a proper geometry of polar or/and charged coordinating groups, but binding affinity must not be so high as to hinder the subsequent release of the ion. The hydrophobic domains of the amphiphile must be located on the surface of the complex to make it soluble in the hydrophobic core of the membrane, whereas the hydrophilic pocket or domain containing the ion must be shielded. Finally, the rate constants of the steps involved, i.e., binding of the ion on one side of the membrane, diffusion of the complex, release of the ion, and return of the ionophore with or without prior binding of a second ion, must be sufficiently high to result in a measurable overall transport rate. These criteria (Pressman, 1976; Reed, 1979; Simon & Carafoli, 1979) form the basis of action of highly specific and potent ionophores of mostly fungal origin, such as valinomycin or A-23187. The criteria have also been used for the rational design and synthesis of ionophores (Simon & Carafoli, 1979). However, given the large number of amphiphilic molecules that are part of general metabolism, it could be expected that some of them will also satisfy, to a greater or smaller degree, the above conditions. These "chance ionophores", if present in excessive amounts, might become detrimental to cells that are exposed to them. The Ca^{2+} ionophoretic activity of phosphatidic acid and of certain oxidized fatty acids (Serhan et al., 1981) might fall into this category.

Bile acids are relatively flat and rigid molecules with a polar and a hydrophobic face; a complex can be envisioned that would consist of two such molecules, with a metal ion sequestered between the two hydrophilic surfaces. Moreover, many bile acids have a relatively high affinity for Ca^{2+} (Jones et al., 1986; Mukidjam et al., 1986; Oelberg et al., 1984). Therefore, the Ca^{2+} ionophoretic properties of bile acids can be rationalized in terms of their structure. These properties have been previously noted by us and by others (Abramson & Shamoo, 1979; Hunt & Jawaharlal, 1980; Maenz & Forsyth, 1984; Child & Rafter, 1986; Oelberg et al., 1987, 1988; Montrose et al., 1988; Anwer et al., 1989) and have been explained by various mechanisms, including channel formation (Abramson & Shamoo, 1979), the formation of inverted micelles (Hunt & Jawaharlal, 1980), and the mobile carrier mechanism (Oelberg et al., 1987, 1988). In contrast to the above results, some workers failed to observe any Ca^{2+} ionophoretic activity of bile acids (Combettes et al., 1989). Therefore, a closer examination of bile acids as potential ionophores in well-defined systems appeared worthwhile. If corroborated, the phenomenon would be of considerable physiological interest because bile acids, present in both plasma and intestinal contents where Ca^{2+} is abundant, could under certain conditions cause an influx of Ca^{2+} into cells exposed to these fluids. For example, this mechanism has been proposed for the bile acid induced intestinal fluid secretion (Maenz & Forsyth, 1984), even though this explanation has been disputed (Ammon et al., 1986), and for hemolysis of red blood cells (Child & Rafter, 1986; Oelberg et al., 1987). An increase of cytoplasmic Ca^{2+} activity in isolated or cultured cells exposed to bile acids has been demonstrated in vitro (Anwer et al., 1989; Montrose et al., 1988).

The purpose of the present work was a further characterization of bile acids as Ca^{2+} ionophores, including specificity, concentration dependence, and mechanism. The methods used were as follows: (i) bile acid dependent extraction of Ca^{2+}

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into a bulk organic phase (Haynes & Pressman, 1974; Pfeiffer & Lardy, 1976), which in itself is an insufficient criterion for establishing ionophoretic activity but yields information on whether the agent under study could act as a mobile carrier as opposed to a channel former, and (ii) direct measurements in vesicles in which a Ca^{2+} gradient was maintained by active pumping. The simplicity of the first system makes it amenable to physicochemical measurements and straighforward mathematical analysis. The second system, while still fully controlled since it is reconstituted from defined components, closely mimics the situation in a cell, rendering the measurements biochemically relevant.

Experimental Procedures

Materials. [3β-^{3}H]-3α-hydroxy-5β-cholanoic acid (tritiated lithocholic acid) was prepared by reduction of methyl 3-oxo-5β-cholanoate with NaB^3H_4 followed by separation of the resulting 3-epimers and hydrolysis of the methyl ester, as described previously for other bile acids (Radominska-Pyrek et al., 1986a). Tauro[^{3}H]lithocholic acid was synthesized from tritiated lithocholic acid by a published procedure (Tserng et al., 1977); the chemical and radiochemical purity of the product was confirmed by thin-layer chromatography in two solvent systems [dichloromethane:2-propanol:acetic acid:water (40:40:4:1 by volume) and ethyl acetate:ethanol:concentrated aqueous ammonia (3:3:1 by volume)] followed by visualization of the spots (Radominska-Pyrek et al., 1986a) or fluorography, respectively. Lithocholic acid was recrystallized (Radominska-Pyrek et al., 1986a) to free it from 5β-cholanoic acid [compare footnote 2 in Radominska-Pyrek et al. (1986a)]. Lithocholic acid 3-*O*-glucuronide was synthesized as described previously (Radominska-Pyrek et al., 1986b); lithocholic acid 3-*O*-sulfate was prepared according to Tserng and Klein (1977). All other reagents, including the taurine conjugates of cholic, deoxycholic, chenodeoxycholic, and lithocholic acids, were analytical-grade commercial products.

Reconstitution. Rabbit skeletal muscle sarcoplasmic reticulum (washed R_1) was obtained by a modification (Zimniak & Racker, 1978) of the method of MacLennan (1970) and was used directly as the enzyme source for reconstitutions. The freeze–thaw sonication technique of reconstitution (Kasahara & Hinkle, 1977), as adapted for the Ca^{2+}-ATPase (Zimniak & Racker, 1978), was used throughout the study. Briefly, 33.3 μL of sonicated asolectin (soybean lipids, Associated Concentrates, exhaustively sonicated at 60 mg/mL in water), 50 μg of sarcoplasmic reticulum protein (except for controls; see below), 50 μL of 0.1 M K^+-HEPES, pH 7.5, and H_2O to 100 μL were combined in a 13 × 100 mm glass test tube. The mixture was frozen in liquid N_2, allowed to thaw for 15 min at room temperature, vortex-mixed, and sonicated for 10 s in a cylindrical ultrasonic bath (Laboratory Supplies Co., Hicksville, NY). The time of sonication and positioning of the tube during sonication were carefully standardized in preliminary experiments to obtain optimal reconstitution (see Results). For larger amounts, multiple tubes were prepared exactly as described above, and the individual reconstitutions were combined after sonication but prior to assay. In controls, the sarcoplasmic reticulum was replaced by buffer (0.3 M sucrose and 5 mM K^+-HEPES, pH 7.5).

Transport Assay. The assay medium included 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM ATP, 0.25 M sucrose, 0.1 mM $^{45}CaCl_2$ (approx. 3×10^4 cpm/mmol), and, where specified, the bile acid under study. All incubations were carried out at 23 °C. Reconstituted vesicles were suspended in the assay medium at 20 μg of protein/mL (0.8 mg of phospholipid/mL) and 200-μL aliquots were withdrawn at predetermined times, usually between 1 min and 2 h. The aliquots were immediately passed through a 0.5 × 6 cm column of Dowex 50W (Sigma Chemical Co.) in Tris form, prepared and handled as described previously [Gasko et al., 1976; see also Zimniak and Racker (1978)]. The radioactivity of the eluate, corresponding to the sum of intravesicular and membrane-bound Ca^{2+}, was determined by liquid scintillation counting. In control experiments in which choline leakage was measured, reconstitution was carried out in the presence of 0.23 mM [^{14}C]choline. The vesicles were subsequently freed from external choline by the Sephadex centrifugation technique (Penefsky, 1977, 1979) and immediately used for incubations. The Dowex column assay (Gasko et al., 1976), identical with that used for Ca^{2+} transport, was employed for the choline controls.

Table I: Partitioning of Calcium and Taurolithocholate between an Unbuffered Aqueous Phase and an Organic Phase Consisting of 1-Butanol:Toluene (3:7)[a]

additions	total calcium concn		total TLC[b] concn	
	organic phase (μM)	aqueous phase (μM)	organic phase (μM)	aqueous phase (μM)
50 μM Ca^{2+}, no TLC	0.09	46.31	0	0
50 μM Ca^{2+}, 10 μM TLC	0.23	46.16	0.69	8.28
50 μM Ca^{2+}, 100 μM TLC	6.10	39.42	17.76	75.74
no Ca^{2+}, 10 μM TLC	0	0	0.26	9.18
no Ca^{2+}, 100 μM TLC	0	0	3.52	89.77

[a] See Experimental Procedures for experimental details. [b] TLC, taurolithocholic acid.

Extraction Assay. Bile acid mediated extraction of Ca^{2+} into a bulk organic phase was measured as follows. The two-phase system consisted of 0.6 mL of water, containing $^{45}CaCl_2$ and other salts as indicated, and 0.6 mL of an organic phase [1-butanol:toluene (3:7 by volume); Haynes & Pressman, 1974; Pfeiffer & Lardy, 1976], containing the tritium-labeled bile acid; typically, the bile acid was dissolved in 1-butanol with the help of sonication, and toluene was added slowly with stirring. The combined aqueous and organic phases were vigorously vortex-mixed for 2 min and centrifuged in a polypropylene tube in a Beckman Microfuge at 8700*g* until phase separation was complete (usually 2–4 min). The separated phases were sampled and the total concentrations of calcium and bile acid were determined by double-label scintillation counting. Deviations from this protocol will be noted below.

Results

The equilibration of a 50 μM solution of $CaCl_2$ with an organic solvent [1-butanol:toluene (3:7)] resulted in only a small amount, less than 0.2%, of the Ca^{2+} being extracted into the organic phase (Table I). Addition of the hydrophobic bile acid sodium taurolithocholate caused a marked increase of Ca^{2+} in the organic phase. This effect was noticeable at 10 μM bile acid and became more pronounced at higher concentrations. On the other hand, the presence of Ca^{2+} also led to a redistribution of taurolithocholate between the two phases. The additional amount of Ca^{2+} that entered the organic layer in the presence of taurolithocholate and the excess of the bile acid that was extracted in the presence of Ca^{2+} remained in an approximate ratio of 1:2 (as calculated from the data shown in Table I). These results are compatible with the formation of a complex consisting of 1 Ca^{2+} ion and 2 taurolithocholate

Table II: Values of the Calcium–Taurolithocholate Complex Formation Constant $K^{a,b}$

pH of aqueous phase	K (M^{-2})
5.0	5×10^7
6.0	2×10^7
7.0	3×10^7
8.0	6×10^7
9.0	6×10^7

[a] See eq 5 in the text; $n = 2$ is assumed. [b] Measured in a two-phase system consisting of 1-butanol:toluene (3:7) and 50 mM MES adjusted with Tris (pH 5–7) or 50 mM Tris adjusted with MES (pH 8 and 9).

molecules. A deeper insight into the process can be gained by considering the partial reactions that are likely to be involved. The protonated form of the bile acid can partition between the aqueous and organic phase:

$$AH_{aq} \rightleftharpoons AH_{org} \quad (1)$$

with a partition coefficient K_1. The dissociation of the bile acid in the aqueous phase

$$A^-_{aq} + H^+_{aq} \rightleftharpoons AH_{aq} \quad (2)$$

has an equilibrium constant K_2 that is approximately 80 M^{-1} for taurine conjugates of bile acids. Finally, the formation of the bile acid–calcium complex at the interphase can be described by

$$Ca^{2+}_{aq} + nAH_{org} \rightleftharpoons nH^+_{aq} + A_nCa_{org} \quad (3)$$

with an equilibrium constant K_3. By multiplying eqs 1 and 2 by n and adding them to eq 3, the final equation is obtained:

$$nA^-_{aq} + Ca^{2+}_{aq} \rightleftharpoons A_nCa_{org} \quad (4)$$

The overall equilibrium constant for this process is

$$K = K_1^n K_2^n K_3 = \frac{[A_nCa]_{org}}{[Ca^{2+}]_{aq}[A^-]^n_{aq}} \quad (5)$$

Equation 5 indicates that K is pH-dependent as long as the pH is kept well above the pK of the bile acid, i.e., above 1.9 for taurine-conjugated bile acids (see discussion below). This is in contrast to a similar equilibrium constant derived for the calcium ionophore A-23187, which is strongly pH-dependent (Pfeiffer & Lardy, 1976). This is due to the fact that, unlike bile acids, the latter ionophore does not partition into the aqueous phase and does not undergo dissociation (Pfeiffer & Lardy, 1976). The results presented in Table II show that, within experimental error, K is in fact independent of the pH of the aqueous phase between 5 and 9. This indicates that eq 4 adequately describes the overall process of calcium complexation. Rearranging of eq 5 yields

$$\log ([A_nCa]_{org}/[Ca^{2+}]_{aq}) = n \log [A^-]_{aq} + \log K \quad (6)$$

The term $[A_nCa]_{org}$ is equal to the total calcium concentration in the organic phase minus the concentration in the absence of a bile acid (Table I); $[Ca^{2+}]_{aq}$ is directly measurable. $[A^-]_{aq}$ is equal to the measurable total concentration of bile acid in the aqueous phase provided the pH is well above the pK of the acid. In our experiments, the pH of the unbuffered aqueous phase was measured to be between 4 and 6, depending on the concentration of the bile acid, and was unaffected by Ca^{2+} at the concentrations used. Since the pK values of taurine-conjugated bile acids are approximately 1.9, the acid was more than 99% ionized in all cases. A Hill plot (not shown) of log $([A_nCa]_{org}/[Ca^{2+}]_{aq})$ versus log $[A^-]_{aq}$ yields a straight line with the slope n, the experimental stoichiometry of the bile acid–calcium complex. The measured slope, 1.75, indicates a stoichiometry of 2 bile acid molecules/calcium ion. The value of K, calculated from the intercept of the regression line, is 2.3×10^6. Because the slope of the line is slightly less than the theoretical value of 2 (see Discussion), the value of K is likely to be underestimated. When K is calculated from eq 5 with $n = 2$, the data from the above plot yield a value of $(3.1 \pm 0.5) \times 10^7$ M^{-2} (mean ± SD). This result, together with additional measurements obtained in unbuffered aqueous phases and taurolithocholate concentrations from 10 to 200 μM as well as in buffered aqueous phases (pH 5–9) and 50 μM bile acid, yields an overall mean value for K of $(5.2 \pm 3.2) \times 10^7$ M^{-2} (15 measurements) if the stoichiometry of 2 is accepted.

Table III: Values of the Complex Formation Constant K^a of Ca^{2+} with Various Bile Acids[b]

bile acid	K (M^{-2})
sodium taurocholate	$(6.3 \pm 3.8) \times 10^4$
sodium taurodeoxycholate	$(6.0 \pm 2.8) \times 10^4$
sodium taurochenodeoxycholate	$(3.3 \pm 2.4) \times 10^5$
sodium taurolithocholate	$(4.6 \pm 3.4) \times 10^7$
sodium lithocholate	$(4.0 \pm 2.4) \times 10^6$

[a] See eq 5 in the text; $n = 2$ is assumed. [b] The partitioning measurements have been carried out in a two-phase system with 1-butanol:toluene (3:7) containing the bile acid (10–300 μM) and an unbuffered aqueous phase containing 50 μM $CaCl_2$.

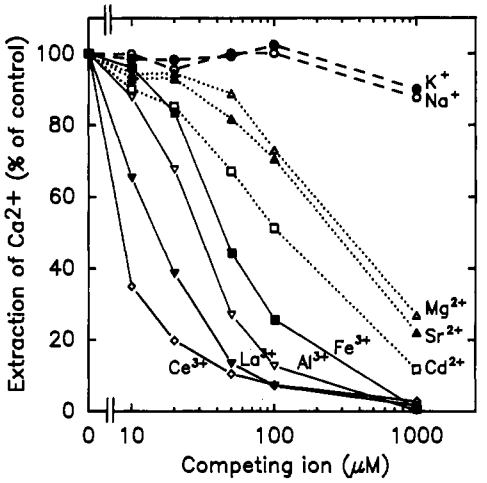

FIGURE 1: Inhibition of taurolithocholate-mediated extraction of Ca^{2+} into an organic phase by other metal ions. The unbuffered aqueous phase contained 50 μM $CaCl_2$ and up to 1000 μM of the ions indicated; except for cerous sulfate, all cations were added as chlorides. The organic phase [1-butanol:toluene (3:7)] contained 100 μM taurolithocholate. See Experimental Procedures for details. Each point represents the mean of 2–7 separate determinations. Monovalent cations are shown by dashed lines, divalent by dotted, and trivalent by solid lines.

The equilibrium constant K is a measure of the affinity of a bile acid for Ca^{2+} and of its ability to extract Ca^{2+} into the bulk organic phase. The constants have been measured for several bile acids and are listed in Table III. The hydrophobicity of a bile acid, as measured by reverse-phase high-pressure liquid chromatography (Armstrong & Carey, 1982), is a good predictor of its ability to transfer Ca^{2+} into the organic phase.

The relative specificity of taurolithocholate for various cations was estimated by competition experiments. The aqueous phase contained, in addition to 50 μM Ca^{2+}, the test ion at concentrations ranging from 10 μM to 1 mM, i.e., up to a 20-fold excess over Ca^{2+}; the organic phase contained 100 μM sodium taurolithocholate. The results are presented in Figure 1. The monovalent ions Na^+ and K^+ did not effectively compete with Ca^{2+}, even if present in large excess. Of the physiologically important divalent cations, Mg^{2+} inhibited the extraction of Ca^{2+} only at high concentrations; if both ions

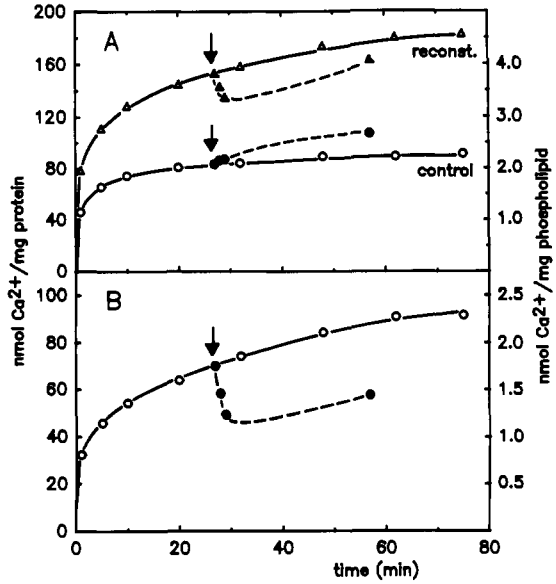

FIGURE 2: Uptake of Ca^{2+} by reconstituted vesicles containing a Ca^{2+}-ATPase. The vesicles were prepared and assayed as described under Experimental Procedures. Panel A: solid lines, reconstituted (Δ) and control (protein-free) (○) vesicles; dashed lines, reconstituted (▲) and control (●) vesicles after addition of 50 μM sodium taurolithocholate; arrow, time of addition of taurolithocholate. Panel B: difference between reconstituted and control vesicles (○) without taurolithocholate and (●) after addition of 50 μM taurolithocholate. A representative experiment (chosen from 12 measurements done at 50 μM taurolithocholate) is shown.

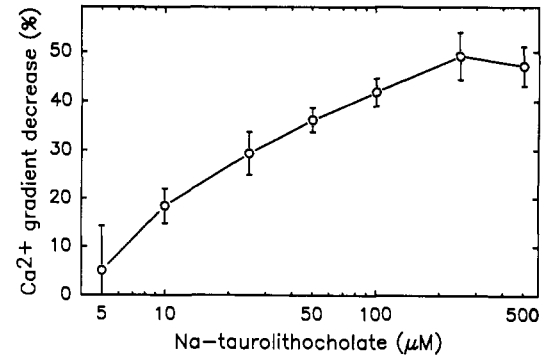

FIGURE 3: Decrease of the magnitude of the steady-state gradient of Ca^{2+} in pumping Ca^{2+}-ATPase vesicles as a function of the concentration of sodium taurolithocholate in the aqueous phase. Means ± SEM (n = 4–12) are shown.

were present at equal concentrations, the extraction of Ca^{2+} was only slightly reduced (by about 10%). Sr^{2+} behaved similarly to Mg^{2+}, whereas La^{3+} competed with Ca^{2+} very effectively, giving approximately 90% inhibition if present at an equal concentration. The order of affinities of various ions for taurolithocholate, as established by their ability to half-inhibit the extraction of Ca^{2+} into an organic phase, was $Ce^{3+} > La^{3+} > Al^{3+} > Fe^{3+} > Ca^{2+} > Cd^{2+} > Sr^{2+} > Mg^{2+} > Na^{+} = K^{+}$ (see Figure 1). It is noteworthy that trivalent ions are more effective than divalent ions and the latter are more effective than monovalent ions. There was no obvious correlation between the crystal ionic radius of the ions and their behavior in the two-phase extraction system. The specificity of the extraction process for Ca^{2+} over Na^{+} and K^{+} is consistent with our previous finding that Na^{+} and Rb^{+} uptake by small unilamellar vesicles was unchanged in the presence of taurodeoxycholate, whereas $^{45}Ca^{2+}$ uptake was promoted (Oelberg et al., 1988), as well as with the finding that bile acids do not extract $^{22}Na^{+}$ into, or carry it across, a bulk organic phase (Accatino & Gavilan, 1988).

Proteoliposomes reconstituted with the Ca^{2+}-ATPase from sarcoplasmic reticulum were able to accumulate Ca^{2+} in the presence of ATP. The time course of Ca^{2+} accumulation by vesicles containing the Ca^{2+} pump and by control (protein-free) vesicles is shown as solid lines in Figure 2A; the difference of these two lines is shown in Figure 2B. The extent of net accumulation after 60 min was about 90 nmol/mg of protein or 2.3 nmol/mg of phospholipid (solid line in Figure 2B). Since no oxalate or other calcium trapping agent was used, most of the intravesicular ion is likely to be in free solution. The internal volume of vesicles obtained by freeze–thaw–sonication is close to 1 μL/mg of phospholipid (Kasahara & Hinkle, 1977); this yields an intravesicular Ca^{2+} concentration of about 2 mM. The extravesicular total Ca^{2+} concentration is 100 μM, resulting in a free Ca^{2+} concentration of 22.3 μM under the conditions used (5 mM Mg-ATP and pH 7.5) (Fabiato & Fabiato, 1979). Thus, the Ca^{2+}-ATPase is able to maintain an approximately 100-fold concentration gradient of Ca^{2+} under conditions close to the steady state of the vesicle system used. Since the magnitude of this gradient is a function of both the influx of Ca^{2+} through the ATPase and the efflux from the vesicles via existing leaks, any increase in the leaks, such as caused by Ca^{2+} ionophores, would be expected to lead to the establishment of a new, lower steady-state gradient. Addition of a large amount of ionophore should collapse the gradient entirely. Indeed, 2 μM A-23187 caused a complete dissipation of the Ca^{2+} gradient (data not shown). Bile acids had a weaker but nevertheless significant effect, as shown by the dashed line in Figure 2B. At the arrow, 50 μM taurolithocholate was added; as a result, the steady-state level of Ca^{2+} in the vesicles was reduced by approximately 35%.

Several controls were carried out to demonstrate the specificity of the above experimental system. In the absence of vesicles, the amount of Ca^{2+} eluted from the Dowex column was less than 1% of the level reached with proteoliposomes at 60 min, and it was not affected by the addition of taurolithocholate. If vesicles were preloaded with 0.23 mM [^{14}C]choline by carrying out the reconstitution in the presence of this solute followed by removal of extravesicular choline, subsequent dilution with the assay medium and incubation for up to 80 min caused no measurable leak of choline; addition of 500 μM taurolithocholate led to a loss of less than 10% of the radioactivity in both control vesicles and vesicles containing the Ca^{2+}-ATPase. This loss was faster than the time resolution of the method used [several seconds; see Zimniak and Racker (1978)] and did not increase after 30 min of incubation with the bile acid. Moreover, the loss of [^{14}C]choline was independent of the concentration of taurolithocholate between 50 and 500 μM. We therefore conclude that the Ca^{2+} efflux in the presence of this bile acid is not due to a generalized leak, as might have been caused by membrane perturbation by a detergent.

The system, as described and verified above, can be used to assess the Ca^{2+} ionophoretic potency of various compounds. As mentioned earlier, the Ca^{2+} ionophore A-23187, used at 2 μM, led to a complete dissipation of the gradient (100% gradient decrease). The effect of various concentrations of sodium taurolithocholate is shown in Figure 3.

In the presence of Ca^{2+}, more taurolithocholate partitions into the bulk organic phase (see third entry in Table I) than in the absence of this divalent ion. It should be possible to observe a similar effect in vesicles. Indeed, the addition of taurolithocholate to control vesicles (i.e., vesicles with no Ca^{2+}-ATPase) in the standard Ca^{2+}-containing assay medium results in a time-dependent increase of Ca^{2+} associated with

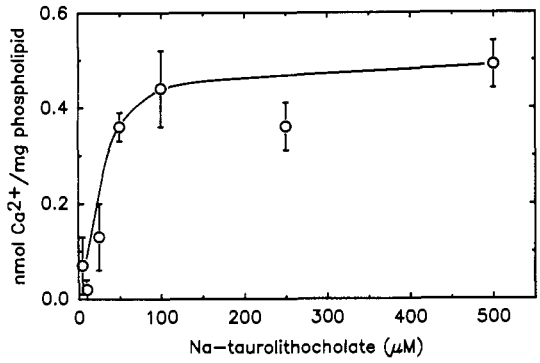

FIGURE 4: Excess (bile acid induced) binding of Ca^{2+} to asolectin vesicles incubated for 30 min in the presence of Ca^{2+} (100 μM total, 22 μM free) and various concentrations of sodium taurolithocholate. Ca^{2+} associated with vesicles in the absence of the bile acid has been substracted from all values. Means ± SEM (n = 4–12) are shown.

the vesicles (lower trace in Figure 2A); by analogy with the bulk organic phase, this Ca^{2+} is presumably present in 1:2 Ca^{2+}/bile acid complexes within the membrane. The same binding of Ca^{2+} is expected to occur in ATPase-containing vesicles. In the latter case, however, it is superimposed on, and overcompensated by, the loss of Ca^{2+} from the intravesicular space (upper line in Figure 2A). The two phenomena can be separated mathematically. The difference between control and reconstituted vesicles (Figure 2B) yields the true loss from the internal space, whereas the extent of association with the membrane can be obtained from the control vesicles (lower trace in Figure 2A). This extent, as measured at 30 min after bile acid addition, is dependent on the taurolithocholate concentration used and appears to reach a pleateau at about 0.5 nmol of Ca^{2+}/mg of phospholipid (Figure 4). If the stoichiometry of 2 bile acid molecules/Ca^{2+} and certain assumptions concerning vesicle size (see Discussion) hold in this case, this number would correspond to a maximum of approximately 10 molecules of bile acid associated with each vesicle in the presence of Ca^{2+} in addition to those already present in the absence of the divalent ion.

Both the depression of the steady-state Ca^{2+} gradient in pumping vesicles and the bile acid promoted association of Ca^{2+} with the phospholipid bilayer are dependent on the structure of the bile acid used (Figure 5). The two above effects are largely parallel. In the series of taurine-conjugated bile acids tested, the monohydroxylated compound taurolithocholate was most effective, followed by the more hydrophilic bile acids; this is in agreement with the sequence observed for bile acid mediated $^{45}Ca^{2+}$ uptake by red blood cells (Oelberg et al., 1987). If the bile acids are ordered according to their ability to bind Ca^{2+} to a phospholipid bilayer, the sequence found is identical with that obtained in the bulk two-phase partitioning experiments (Table III). This is consistent with the notion that, in both cases, the underlying process is the formation of a 2:1 bile acid/Ca^{2+} complex and its partitioning into a hydrophobic phase, which can be the core of the membrane or the bulk organic solvent.

Discussion

In the present work we have shown that taurine conjugates of bile acids are able to extract Ca^{2+} from an aqueous to a bulk organic phase. The experimentally found stoichiometry of the extracted complex was 1.75. This points to an electroneutral complex of two bile acid molecules per Ca^{2+} (Figure 6), although the presence of smaller amounts of the "acid salt" (a 1:1 complex), as found for calcium taurocholate in dilute aqueous solution (Mukidjam et al., 1986) and for calcium

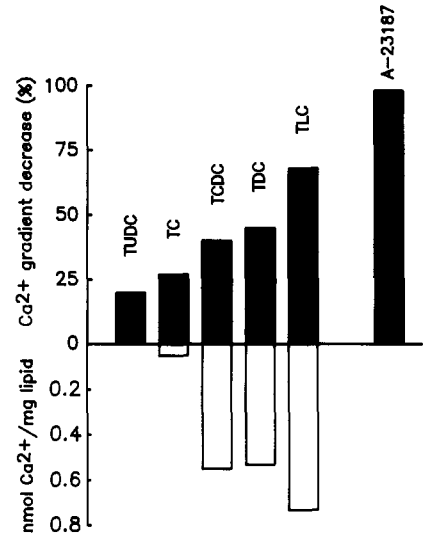

FIGURE 5: Ca^{2+} ionophoretic activity (solid bars) and ability to bind Ca^{2+} to a phospholipid bilayer (open bars) of various bile acids (used at 500 μM) and the ionophore A-23187 (2 μM). Abbreviations used: TUDC, tauroursodeoxycholate; TC, taurocholate; TCDC, taurochenodeoxycholate; TDC, taurodeoxycholate; TLC, taurolithocholate.

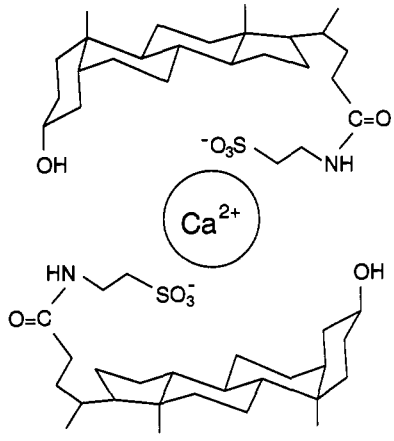

FIGURE 6: Hypothetical structure of the 2:1 taurolithocholate/calcium complex, which is soluble in organic solvents and associates with lipid bilayers.

cholate crystals (Hogan et al., 1984), cannot be ruled out. The bile acids studied exhibit a similar behavior when the bulk organic phase is substituted with a phospholipid bilayer. Ca^{2+} becomes associated with the membrane in a time-dependent manner. Even though we have not directly determined the nature of this process, a partitioning of Ca^{2+}/bile acid complexes into the hydrophobic core of the membrane appears to be the most likely possibility.

The extraction of ions from the aqueous into an organic phase (Haynes & Pressman, 1974; Pfeiffer & Lardy, 1976) by an agent is usually considered a necessary but not sufficient condition in proving ionophoretic properties of that agent. Thus, direct measurements of ion transport across phospholipid bilayers were undertaken. Experiments of this type are often based on the measurement of ion efflux or influx from/into sonicated liposomes in the presence and absence of the compound under study and are hampered by the small internal volume of the vesicles (typically 1 μL/mg of phospholipid). Weak ionophores are not easily distinguished from background leaks, and effective compounds often equilibrate the ion faster than the time resolution of the assay. Spectral techniques can overcome the latter problem, but their sensitivity is still limited by the small internal volume of the vesicles. We have therefore chosen a different technique, based on measurements of

changes in the steady-state ion concentration. An ion pump is incorporated into the vesicles and is used to maintain a steady-state ion gradient whose level depends on the relative magnitudes of pumping and leaks. Once the gradient stabilizes, the leaks can be increased by the addition of an ionophore, and the resulting new steady-state gradient can be measured. The system has two major advantages over the simple mediated diffusion in liposomes: (i) it is sensitive to minor changes in permeability that would be otherwise difficult to detect and (ii) since the system, after perturbation, establishes a new steady state that is easily measurable, rapidly acting ionophores can be assayed with conventional methods without the need for subsecond time resolution.

The particular system employed in this work consisted of the Ca^{2+}-ATPase from sarcoplasmic reticulum incorporated into asolectin liposomes (Zimniak & Racker, 1978). The steady-state Ca^{2+} gradient attained by these vesicles was about 100-fold, in good agreement with the value obtained by measuring the electrical activity of the pump in a similar experimental system (Zimniak & Racker, 1978). In resting muscle, the Ca^{2+} pump is capable of equilibrating the Ca^{2+} gradient with the available driving force (the [ATP]/[ADP][P_i] quotient) (Tanford, 1981). This results in a gradient that is at least 10000-fold (Hasselbach & Oetliker, 1983; Tanford, 1981). This results in a gradient that is at least 10000-fold (Hasselbach & Oetliker, 1983; Tanford, 1981). The lower gradient in the reconstituted vesicles could be due to several factors. (i) The available $\Delta G'$ of the ATP $\rightleftharpoons$ ADP + P_i system could be low. This is unlikely because, with 5 mM ATP added, the ATP would have to be 99.98% hydrolyzed to sustain an only 100-fold gradient at equilibrium. (ii) The external free Ca^{2+} concentration was approximately 2×10^{-5} M (see Results), much higher than the cytoplasmic Ca^{2+} concentration in the resting muscle (Hasselbach & Oetliker, 1983). Even at a 100-fold gradient, the intravesicular Ca^{2+} concentration would already reach 2 mM and could inhibit the pump by binding to the low-affinity site (Inesi, 1985). Thus, the gradient would become self-limiting. (iii) Leaks are already present in the reconstituted vesicles, removing the system from equilibrium and establishing a steady-state Ca^{2+} circulation. Although this would lower the sensitivity of the system to added ionophores, it does not in any way interfere with the principle of the described assay.

If a bilayer thickness of 4 nm and an average surface area of 0.7 nm^2/phospholipid molecule is assumed (Mimms et al., 1981), it can be calculated that a vesicle with a diameter of 40 nm would consist of approximately 12000 phospholipid molecules and have an internal volume of 1.1 μL/mg of phospholipid, the latter being close to the experimental value given for this type of vesicle (0.98 μL/mg; Kasahara & Hinkle, 1977). If vesicles with a diameter of 40 nm are taken to be typical for the present reconstitution, it can be further calculated that at 10 μM taurolithocholate, the lowest concentration that was unequivocally effective in releasing intravesicular Ca^{2+}, the overall average number of bile acid molecules per vesicle was about 120. We have not determined how many of these molecules partition into the hydrophobic core of the membrane since a distinction between the latter and an association with the polar headgroups of the phospholipids would be experimentally difficult. If the partition coefficient of taurolithocholate between water and the 1-butanol/toluene mixture can be applied here (last two entries in Table I), it could be expected that, in the absence of Ca^{2+}, only 4–5 bile acid molecules would dissolve in the hydrophobic core of the membrane of each vesicle, a number that is unlikely to lead to major structural perturbation of the bilayer. This notion is compatible with the finding that the ionophoretic effect of taurolithocholate is specific rather than being due to a generalized leak.

In addition to being able to extract Ca^{2+} from an aqueous into an organic phase, the bile acids studied fulfill the four more stringent criteria established for Ca^{2+} ionophores (Serhan et al., 1981): (1) they translocate Ca^{2+} from one aqueous phase, across a phospholipid bilayer, into a second aqueous phase, (2) they act at micromolar concentrations (or at a total concentration of about 1 mol % of the lipids present and probably at actual concentrations in the bilayer as low as 0.05 mol %, which amounts to 4–5 molecules per vesicle), (3) they show selectivity for Ca^{2+} over Mg^{2+}, as measured in the two-phase extraction system, and (4) they do not lyse the membrane at the concentrations used. Thus, bile acids can be regarded as true Ca^{2+} ionophores.

The structural basis of the Ca^{2+} ionophoretic activity of bile acids is not entirely clear. Barnes and co-workers have shown in a series of elegant studies (Hogan et al., 1984; Mukidjam et al., 1986) that trihydroxylated bile acids form, in aqueous solution or in calcium–bile acid crystals, a 1:1 complex with Ca^{2+}. The metal ion interacts solely with the acidic group on the side chain; the remaining coordination sites of the metal are occupied by water, and the second charge is neutralized by a small anion such as Cl^-. According to our results, the stoichiometry of the complex would change to 2:1 upon extraction into an organic phase. This could provide for a more efficient shielding of the Ca^{2+} ion from the apolar medium (Figure 6). The possible involvement of hydroxyl groups of the bile acid in a 2:1 complex remains open. The facts that 5β-cholanoic acid, which lacks hydroxyl groups on the steroidal nucleus, was inactive in the two-phase partitioning system and that the 3-*O*-glucuronide and 3-*O*-sulfate of lithocholic acid extracted considerably less Ca^{2+} than lithocholic acid itself (data not shown) could be taken as evidence that the presence and proper geometry of hydroxyl groups is essential for complex formation. However, other factors could also play a role. In the case of the two latter bile acid derivatives, both the carboxyl group of the side chain and the acidic group of the glucuronic or sulfuric acid could participate in coordination of the Ca^{2+} ion. If so, the resulting 1:1 complex would have a polar, unshielded surface and may not partition efficiently into the organic phase. The question whether, in the 2:1 complex with bile acids such as taurolithocholate, Ca^{2+} remains at least partially hydrated and whether the hydroxyl groups of the bile acid participate in its coordination would require structural studies that are beyond the scope of the present work. In any case, bile acids are likely to function as mobile (in contrast to channel-forming) Ca^{2+} ionophores, with the 2:1 bile acid/Ca^{2+} complex as the possible transport intermediate, as indicated by the species that is extracted into a bulk organic phase.

The ionophoretic activity of the bile acids has been demonstrated under conditions that mimic closely the in vivo situation at the plasma membrane of a cell: in both cases, a Ca^{2+} pump maintains a steady-state gradient of Ca^{2+} across the membrane. This would imply that bile acids, if present at sufficient concentrations in body fluids, could exert their effects on a variety of cells by increasing intracellular Ca^{2+} concentrations. In fact, using fluorescent Ca^{2+} indicators, we were able to demonstrate an increase in cytoplasmic Ca^{2+} activity in isolated hepatocytes suspended in a Ca^{2+}-containing medium and exposed to taurolithocholate (Anwer et al., 1989), as well as in cultured renal epithelial cells under similar

conditions (Montrose et al., 1988). Others (Combettes et al., 1988, 1989) have demonstrated a bile acid triggered increase in cytoplasmic Ca^{2+} concentration due to Ca^{2+} release from an intracellular inositol 1,4,5-triphosphate sensitive pool, although in this case the mechanism of action of bile acids may not have been related to their Ca^{2+} ionophoretic properties and the phenomenon was restricted to hepatocytes (Coquil et al., 1991). Both mechanisms, namely, release of Ca^{2+} from intracellular stores and bile acid mediated influx across the plasma membrane, may coexist. Bile acid concentrations in body fluids appear to be adequate for triggering of Ca^{2+} influx into the cytoplasm, at least in some situations. Plasma levels of bile acids in the human, close to 5 μM in the postprandial and 2 μM in the fasting state (Hedenborg et al., 1985), are not sufficient to cause a marked Ca^{2+} influx into the cell, especially since the ionophoretically most potent monohydroxylated bile acid, lithocholate, constitutes only a small percentage of the total (5–10%; Hedenborg et al., 1985). However, the levels can rise dramatically in liver disease. For example, bile acid concentrations close to 400 μM, including taurochenodeoxycholate in excess of 200 μM, have been reported in patients with acute hepatitis (Takikawa et al., 1986); at this concentration, taurochenodeoxycholate has a significant ionophoretic effect (see Results).

We have shown that hydrophobic bile acids behave as Ca^{2+} ionophores and thus have the capacity to raise intracellular Ca^{2+} levels. A clear demonstration that such a bile acid mediated effect can alter or derail cellular homeostasis in vivo is still outstanding. One attempt of such demonstration was unsuccessful: in a perfused liver, bile acid concentrations that led to cholestasis failed to increase the intracellular Ca^{2+} concentration, as determined by indirect methods (Farrell et al., 1990). However, more sensitive and/or specific methods of measuring intracellular Ca^{2+} concentration may be required. Alternatively, excessive amounts of bile acids, as seen in many liver diseases, could directly influence membranes via the accumulation of the bile acid/Ca^{2+} complex. We therefore believe that bile acid mediated calcium effects could play a causative role in many side effects observed in liver disease.

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