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Bile salt-induced calcium fluxes in artificial phospholipid vesicles

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The ionic permeability of selected biological membranes is increased by bile salts. To examine changes in calcium permeability during the exposure of artificial membranes to bile salts, we investigated calcium uptake by unilamellar and multilamellar phospholipid vesicles. In the presence of 750 μM taurodeoxycholate, uptake of radiolabelled calcium by unilamellar vesicles increased 2.5-fold over control values. Calcium uptake by multilamellar vesicles as measured with a free calcium indicator, arsenazo III, increased 2.2- or 21-fold in the presence of 60 μM lithocholate or 3β-hydroxy-5-cholenoate, respectively. Results were directly influenced by experimental variables such as bile salt hydrophobicity, external calcium concentration, and the bile salt/lipid molar ratio. Observed membrane solubilization was minimal despite increased calcium permeability. Comparison of radiolabelled calcium uptake with radiolabelled sodium or radiolabelled rubidium uptake indicated that bile salt-dependent calcium uptake was 60–140-times greater than bile salt-dependent uptake of either monovalent cation. In an effort to delineate forces affecting calcium translocation, vesicles were exposed either to valinomycin, which induced an electrochemical gradient across the membrane, or to nigericin, which induced a proton gradient. Exposure to valinomycin minimally influenced bile salt-induced calcium uptake while exposure to nigericin significantly promoted uptake by 40–70%. The results suggest that bile salts promote calcium uptake by a mechanism which may be similar to those of other carboxylic ionophores.

Introduction

Previous studies have suggested that bile salts increase the permeability of selected membranes to divalent and monovalent cations [1–4]. The mechanism(s) by which these ionic permeability changes occur, however, is not understood. It is clear that at concentrations exceeding 10^{-3} M, certain bile salts become potent detergents which are capable of solubilizing membrane components [5]. Exposure of intact cellular systems to 10^{-3} M bile salt concentrations rsults in both the solubilization of membrane-associated enzymes and a decline in metabolic activities which correlate with the general disruption of the cell membrane [6]. Hence, one proposed mechanism of bile salt-induced changes in membrane permeability relates to the disruption of membrane integrity and co-

Abbreviations: SUV, small unilamellar vesicle; MUV, multilamellar vesicle; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; arsenazo III, 2,7-bis(arsonophenylazo)-1,8-dihydroxynaphthalene-3,6-disulfonic acid.

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micellization of membrane lipids. Of somewhat greater interest to us, however, are the observations of bile salt-induced permeability changes at bile salt concentrations substantially lower than those expected to solubilize membrane components [2–4]. Because hydrophobic bile salts are more effective than hydrophilic bile salts at promoting these activities, another mechanism involving the movement of bile salts into membranes is suggested. Movement into membranes, in part, is related to hydrophobicity of the bile salt molecule. It is proposed, therefore, that bile salts partition into membranes and alter cation permeability by either introducing pores or acting as mobile carriers.

To date, most studies regarding bile salt-induced permeability changes have focused upon the influence of bile salts on calcium translocation. Abramson and Shamoo initially demonstrated bile salt-induced movement of calcium across black lipid membranes [1]. More recently, bile salt-promoted influx or efflux of radiolabelled calcium across vesicular and cellular membranes has been measured [3,4,7,8]. Interpretation of the results, however, is complicated by the possible uncontrolled influence of bile salts on membrane pump activities or nonspecific membrane permeabilities. Thus, it remains unclear whether bile salts promote calcium uptake through a direct effect upon calcium transport, as observed for conventional calcium ionophores, or through effects upon any of several membrane processes that indirectly influence calcium uptake.

To resume investigation of this problem, the present study was conducted employing two different systems of artificial phospholipid vesicles. In the first system calcium accumulation by small unilamellar vesicles (SUVs) prepared from soy phospholipids was monitored employing well-established radiolabelled calcium methodology. In the second system, the free calcium in multilamellar vesicles (MLVs) prepared from purified lipids was monitored spectrophotometrically as in studies of calcium transport induced by substances such as phosphatidic acid, oxidized fatty acids, and leukotrienes [9,10]. Employing the above two systems, membrane permeability to calcium in the presence of bile salt was compared to permeability under control conditions. These studies provide evidence that selected bile salts have a 2-fold effect upon membranes. (i) At high concentrations bile salts induce a generalized increase in membrane permeability secondary to detergent action at membrane sites. (ii) At lower concentrations, bile salts preferentially increase calcium permeability by mechanisms similar to those of more conventional calcium ionophores.

These results were presented in part at the Society for Pediatric Research Meeting in Washington, DC, May, 1985, and have been published in abstract form [11].

Methods

Preparation of phospholipid vesicles

Preparation of SUVs. SUVs were prepared by sonication. 50 mg of soy asolectin were added to 1.0 ml of 50 mM citric acid titrated to pH 7.4 with KOH. The mixture was sonicated in a 13×100 mm pyrex tube in a bath type sonicator (General Laboratory Supplies, Hicksville, NY) for 10 min at room temperature. The vesicle suspension was centrifuged ($10000 \times g$) for 10 min to remove phospholipid crystals and then passed through a 1.6×20 cm Sephadex G-25 medium grade column which was equilibrated with 200 mM mannitol and 10 mM Tris-Hepes, pH 7.4. Passage through the column replaced external potassium citrate with mannitol, thus creating a potassium gradient across the vesicle membrane [12] which, with the use of specific ionophores, could be used to generate a membrane potential or pH gradient.

Preparation of MLVs loaded with arsenazo III. MLVs were prepared from purified phospholipids by the method of Bangham et al. [13]. Purified phosphatidylcholine, (23.6 μmol), dicetyl phosphate, (6.8 μmol) and cholesterol (3.4 μmol) were dissolved in chloroform. Chloroform was evaporated off under vacuum before 1.0 ml buffer solution (145 mM KCl, 10 mM Na-Hepes, pH 7.4) and 3 μmol of calcium-free sodium-arsenazo III were added. The mixture was vortexed at moderate speed for 20 min and equilibrated for 2 h at room temperature. Subsequently, the mixture was passed through a 1.5×27 cm Sephadex G-50 fine grade column equilibrated with the above KCl buffer to separate intra- and extravesicular fractions of arsenazo III [14]. Under these conditions

MLV-associated arsenazo III eluted several minutes before the free compound. The percentage of entrapped arsenazo III as determined from the comparison of μmol of loaded arsenazo III to μmol of unentrapped arsenazo III was 24.5 $\pm$ 0.8%. Captured volume and entrapment efficiency calculated from initial [arsenazo III], percentage of entrapped arsenazo III, and MLV lipid content were 7.7 $\pm$ 0.5 l/mol and 2.7 $\pm$ 0.1%, respectively [3].

Phosphatidylcholine content of SUVs and MLVs

Lipids were extracted from vesicle aliquots by the method of Bligh and Dyer [15]. The phosphatidylcholine content of the lipid extract was assayed by colorimetric determination with $NH_4Fe(SCN)_3$ by the method of Stewart [16]. We determined that dicetyl phosphate did not interfere with the assay. The calibration curve was linear over a range of 0–0.08 mg phosphatidylcholine ($r = 0.999$).

Spectrophotometry of arsenazo III

The commercially available sodium salt of purified arsenazo III was further purified by the method of Weissmann et al. employing cation exchange resin Chelex 100 and acidic recrystallization [14]. Differential absorbance measurements between the free arsenazo III and the arsenazo III-Ca complex were performed at paired wavelengths of 656 and 700 nm ($\Delta A_{656-700}$) with a dual-wavelength time-sharing spectrophotometer (Model MB2/TSF1 Spectrophotometer, Johnson Resarch Foundation, Philadelphia, PA). The differential coefficient of absorbance ($\Delta\epsilon = 15.74$ $mM^{-1} \cdot cm$) between arsenazo III and arsenazo-Ca and the dissociation constnat ($K_d = 49.3$ μM) for arsenazo III-Ca were calculated from $\Delta A_{656-700}$ values resulting from arsenazo III and Ca titrations, respectively, of buffered EGTA solutions with known calcium concentrations [17]. The K_d value was unaffected by the presence of bile salt.

MLVs with incorporated arsenazo III were prepared freshly each day. Free calcium concentrations were calculated from the arsenazo III-Ca binding model of Brown and Rydqvist [18]. The complexities associated with arsenazo III-Mg binding were avoided by excluding magnesium ion from all buffers.

Transport assays

Cation transport by SUVs was assessed by the accumulation of radiolabelled solutes. Unless otherwise stated, transport was assayed by the addition of 100 μl of the desalted vesicle suspension to 2.0 ml of buffer solution composed of 116 mM NaCl, 0.9 mM $MgSO_4$, 15 mM Na-Hepes (pH 7.4), 5.00 mM $^{45}CaCl_2$, and the indicated concentration of sodium taurodeoxycholate. Choline was substituted for sodium as the predominant buffer cation in assays comparing radiolabelled monovalent cation uptake with ^{45}Ca uptake. At indicated times 100 μl aliquots of the reaction mixture were eluted through a Dowex cation exchange column by a modification of the proceudre of Gasko et al. [19]. The columns were prepared by pouring 1.5 ml of Dowex 50W-X8, 50–100 mesh, Tris form into a Pasteur pipet with a dacron wool plug. The samples were eluted from the column with 1.9 ml of 300 mM mannitol, collected in scintillation vials, mixed with Liquiscint (National Diagnostics, Somerville, NY), and counted for radioisotopic content with a Tracor Mark III, model 6882, liquid scintillation system (Tracor Analytic, Elk Grove Village, IL). Other additions were as indicated in the results section.

Calcium transport into MLVs was assessed by the time course of free calcium concentration changes as calculated from $\Delta A_{656-700}$ measurements. Following Sephadex column chromatography to remove unincorporated arsenazo III, 1.00 ml of the eluent fraction containing MLVs in 145 mM KCl and 10 mM Na-Hepes (pH 7.4) was placed in a cuvette with 1.00 cm light path. Concentrated bile salt solutions were added as indicated in the Results section. Assays were initiated by the addition of concentrated calcium solution providing 1 mM final calcium concentration. Continuous recordings of $\Delta A_{656-700}$ were maintained over 20 min. Each set of experiments included bile salt-free controls. The contribution of extravesicular arsenazo III-Ca to total $\Delta A_{656-700}$, as evidenced by the immediate rise in $\Delta A_{656-700}$ upon addition of 1 mM calcium, was subtracted from all calculations of free calcium concentration. In all experiments extravesicular arsenazo III-Ca contributed less than 8% to total $\Delta A_{656-700}$.

Release of intravesicular markers

The release of impermeant radiolabelled choline from SUVs was evaluated by passing 100 μl aliquots of SUVs loaded with [^{14}C]choline through Dowex cation exchange columns at indicated time points. SUVs were eluted from the columns and counted by the liquid scintillation technique as described for measurements of radiolabelled calcium.

Release of arsenazo III from MLVs exposed to bile salt and calcium was determined by comparison of $\Delta A_{656-700}$ values before and after elution through a Sephadex column. Columns were prepared by pouring 1.5 ml of Sephadex G-50, fine grade in 145 mM KCl, 10 mM Na-Hepes, (pH 7.4) into a Pasteur pipet with a dacron wool plug. Elution through Sephadex separated reaction mixture aliquots into distinct fractions of entrapped and free arsenazo III; arsenazo III released was determined by comparing $\Delta A_{656-700}$ values at the beginning and end of 20 min incubations.

Materials

Soy bean asolectin was obtained from Associated Concentrates, Woodside, NY. Purified lipids, Hepes, Tris, 3β-hydroxy-5-cholen-24-oic acid, and sodium salts of arsenazo III, taurocholate, taurodeoxycholate and lithocholate were available from Sigma, St. Louis, MO. Valinomycin, nigericin, and sodium salts of cholate, chenodeoxycholate and deoxycholate were purchased from Calbiochem-Behring, La Jolla, CA; Sephadex G-25 and G-50 from Pharmacia Fine Chemicals, Piscataway, NJ; and Dowex 50W-X8 and Chelex 100 from Bio-Rad Laboratories, Richmond, CA. Carrier-free $^{45}CaCl_2$ (25.2 mCi/mg) was obtained from ICN, Irvine, CA and carrier-free ^{86}RbCl (1.6 mCi/mg) and ^{22}NaCl (120 Ci/g) from New England Nuclear, Boston, MA.

Results

Bile salt effects on membrane integrity

Bile salts are recognized as potent detergents which solubilize phospholipid bilayers by the formation of mixed micelles. To evaluate the concentration effect of taurodeoxycholate on SUV membrane integrity the release of impermeant radiolabelled solutes was measured. SUVs were exposed to a range of taurodeoxycholate concentrations (0–3.00 mM) which at the highest concentration exceeded the anticipated critical micellar concentration. [^{14}C]Choline retention in the presence of 5 mM calcium was within 80% of control values following 10 min of exposure to up to 750 μM taurodeoxycholate (89.9 ± 1.4, 87.9 ± 1.4, and 71.0 ± 1.9% retention at 0, 250 μM, and 750 μM taurodeoxycholate, respectively). Substantially less retention of [^{14}C]choline was observed at 3 mM taurodeoxycholate (52.3 ± 9.4% retention). Comparison of the above with ^{86}Rb-loaded SUVs yielded similar results. Based on these findings we recognized that significant disruption of SUVs at elevated bile salt concentrations would reduce apparent calcium uptake as measured by the radiolabelled calcium assay. Accordingly, maximal bile salt concentrations in subsequent studies of SUVs were limited to 750 μM.

The retention of arsenazo III by MLVs in 1 mM calcium was similar to choline retention by SUVs at lower concentrations of taurodeoxycholate (99.1 ± 1.0, 96.3 ± 0.1, and 83.5 ± 1.8% retention at 0, 250, and 500 μM taurodeoxycholate, respectively). At slightly higher bile salt concentrations, retention of arsenazo III by MLVs dropped to less than 65% (57.3 ± 6.0 and 62.7 ± 7.4% retention at 750 μM and 1.00 mM taurodeoxycholate, respectively). To insure minimal leakage of arsenazo III from MLVs, retention was reexamined at bile salt concentrations less than or equal to 120 μM. Under these conditions, and in the presence of lithocholate, which was substituted for taurodeoxycholate in the majority of subsequent MLV studies, mean arsenazo III retention was greater than 99% (99.6 ± 0.4, 99.6 ± 0.4, 99.8 ± 0.2, and 99.2 ± 0.8% retention for 0, 30, 60, and 120 μM lithocholate, respectively). Except where noted, subsequent MLV studies employed 60 μM bile salt concentrations.

Bile salt-dependent calcium accumulation

In the absence of bile salts, SUVs exhibited time-dependent uptake of radiolabelled calcium (Fig. 1). The presence of the bile salts taurocholate or taurodeoxycholate increased the initial rate of calcium accumulation 1.7- and 3-fold, respectively. Ca accumulation plateaued at maximal levels after approx. 60 min at 37°C. Because of the above differences between taurodeoxycholate

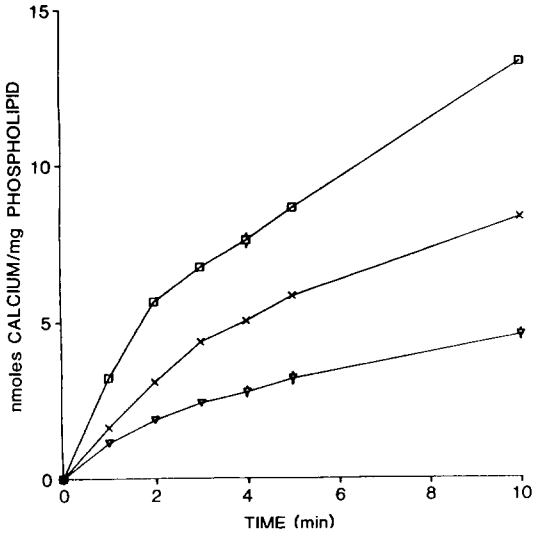

Fig. 1. Bile salt-induced calcium accumulation by SUVs. Vesicles were prepared in 50 mM potassium citrate (pH 7.4). The incubation medium was 116 mM NaCl, 0.9 mM $MgSO_4$, 15 mM Na-Hepes, 5.00 mM $^{45}CaCl_2$, and 750 μM sodium taurocholate (×) or sodium taurodeoxycholate (□). Control experiments (▽) were without bile salt. Each point represents the mean uptake of ^{45}Ca by duplicate assays from four experiments.

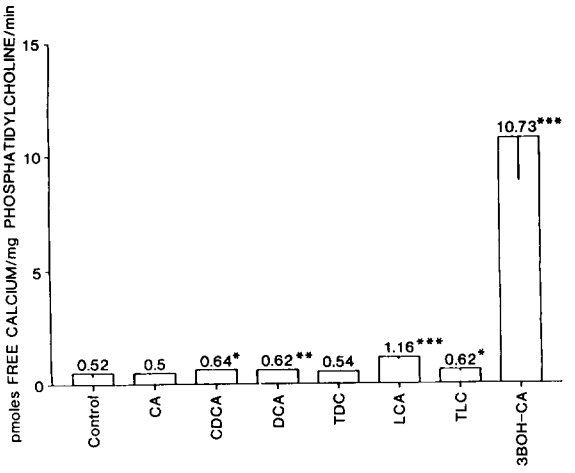

Fig. 2. Bile salt-induced free calcium accumulation by MLVs. Vesicles were prepared in 145 mM KCl, 10 mM Na-Hepes (pH 7.4) and 3 mM arsenazo III. The incubation medium was 145 mM KCl, 10 mM Ha-Hepes (pH 7.4) and 3 mM arsenazo III. The incubation medium was 145 mM KCl, 10 mM Na-Hepes (pH 7.4), 1.00 mM $CaCl_2$ and 60 μM cholate (CA), chenodeoxycholate (CDCA), deoxycholate (DCA), taurodeoxycholate (TDC), lithocholate (LCA), taurolithocholate (TLC), or 3β-hydroxy-5-cholenoate (3BOH-CA). Control experiments were without bile salt. Each bar represents the mean initial rate of free calcium change as indicated from absorbance measurements in four experiments. Vertical lines indicate S.E. values. Asterisks indicate significant differences from control (* $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$).

and taurocholate, subsequent observations of bile salt-dependent calcium accumulation by SUVs employed taurodeoxycholate.

The effect of bile salts upon free calcium accumulation by MLVs was systematically investigated at room temperature in the presence of 60 μM cholate, chenodeoxycholate, deoxycholate, taurodeoxycholate, lithocholate, taurolithocholate, and 3β-hydroxy-5-cholenoate. Prior to bile salt or calcium additions intravesicular free calcium concentration averaged 1.82 ± 0.10 μM (6.64 ± 0.37 pmol/mg phosphatidylcholine). The addition of any of the several 60 μM bile salts prior to the addition of calcium did not change the baseline free calcium concentration. However, the addition of 1 mM calcium increased intravesicular free calcium concentrations in a manner dependent upon the individual bile salt present (Fig. 2). The change in intravesicular free calcium concentration, expressed as the initial rate of free calcium rise over the first 2 min, was greatest for the hydrophobic bile salts 3β-hydroxy-5-cholenoate and lithocholate (10.73 ± 1.93 and 1.16 ± 0.05 pmol/mg phosphatidylcholine per min, respectively). Chenodeoxycholate and deoxycholate promoted intermediate values of free calcium uptake (0.64 ± 0.02 and 0.62 ± 0.01 pmol/mg phosphatidylcholine per min, respectively), while cholate (0.50 ± 0.03 pmol/mg phosphatidylcholine per min) had no apparent effect upon the control rate of uptake (0.52 ± 0.08 pmol/mg phosphatidylcholine per min). Taurine conjugation of deoxycholate and lithocholate reduced the promotional activity of both parent bile salts by at least 15%. Based on the above, all subsequent observations of MLVs employed lithocholate with which to investigate bile salt-dependent calcium accumulation.

Effect of calcium, bile salt, or lipid concentration upon accumulation

The effect of external calcium concentration upon the initial rate of calcium uptake by SUVs or MLVs was investigated at fixed concentrations of

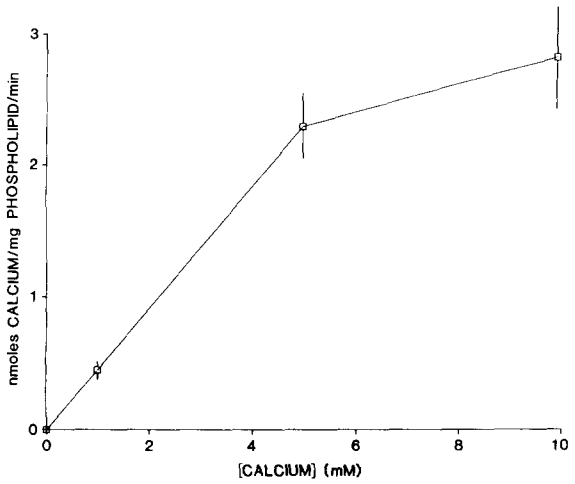

Fig. 3. Bile salt-induced calcium accumulation by SUVs as a function of calcium concentration. The incubation medium was supplemented with 750 μM taurodeoxycholate and 1, 5 or 10 mM $^{45}CaCl_2$. The mean initial rates of ^{45}Ca uptake were determined by duplicate assays from five experiments.

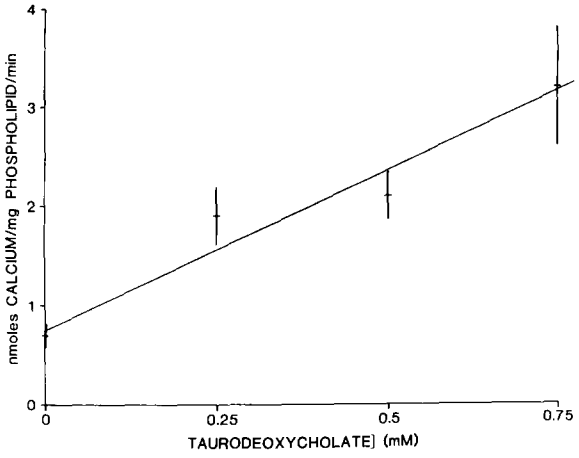

Fig. 5. Bile salt-induced calcium accumulation by SUVs as a function of taurodeoxycholate concentration. The incubation medium was supplemented with 5 mM $^{45}CaCl_2$ and 0, 250, 500 or 750 μM taurodeoxycholate. Each point indicates the mean initial rate of ^{45}Ca uptake by SUVs. The correlation coefficient of the linear regression analysis is 0.83.

taurodeoxycholate (750 μM) or lithocholate (60 μM), respectivley. In both systems the rate of calcium uptake was directly related to an external calcium concentration less than 4–5 mM (Fig. 3 and 4). At concentrations greater than 4–5 mM rates of uptake plateaued, suggesting saturation of the uptake process.

The effect of taurodeoxycholate concentration upon calcium uptake by SUVs was also examined at different taurodeoxycholate concentrations of less than or equal to 750 μM. Within this range of concentrations, calcium uptake was proportional to bile salt concentration (Fig. 5). Although not indicated in the figure, higher concentrations of taurodeoxycholate (3 mM) decreased apparent

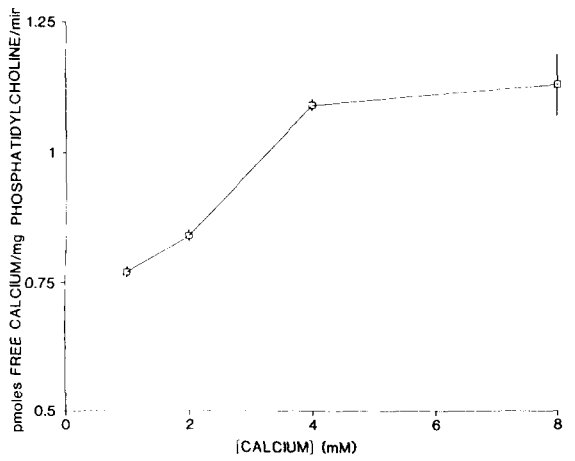

Fig. 4. Bile salt-induced free calcium accumulation by MLVs as a function of calcium concentration. The incubation medium was supplemented with 60 μM lithocholate and 1, 2, 4 or 8 mM $CaCl_2$. Each point is the mean initial rate of free calcium change in MLVs.

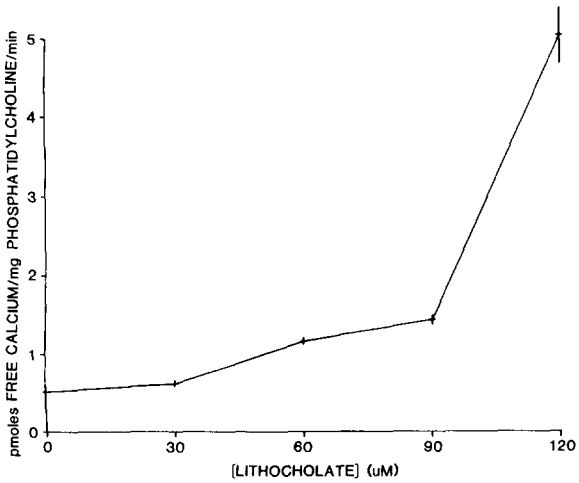

Fig. 6. Bile salt-induced free calcium accumulation by MLVs as a function of lithocholate concentration. The incubation medium was supplemented with 1 mM $CaCl_2$ and 0, 30, 60, 90 or 120 μM lithocholate. Each point indicates the mean initial rate of free calcium change in MLVs.

calcium uptake as solubilization of vesicles reduced membrane integrity.

The shape of the dose-response curve of MLVs to increasing lithocholate concentrations differed from that of SUVs (Fig. 6). Between 30 and 90 μM, calcium uptake was directly related to lithocholate concentration. However, between 90 and 120 μM there was a marked increase in calcium uptake. This nonlinear response of calcium uptake to increasing lithocholate concentrations suggested a possible relationship between calcium uptake and the lithocholate/lipid molar ratio. To investigate this possibility, the calcium response to varying concentrations of MLVs was determined while external calcium (1.0 mM) and lithocholate (60 μM) concentrations were held constant. By this design, free calcium uptake in the presence of bile salt was inversely related to MLV concentration in an nonlinear manner (Fig. 7). The inflection point of the curve occurred at a lithocholate/lipid molar ratio of 0.036. This value was similar to the lithocholate/lipid molar ratio at the inflection point of Fig. 6 (0.030). Calcium uptake in the absence of bile salt was unaffected (Fig. 7). Intersection of the two curves in Fig. 7, at which point the rates of calcium uptake in the presence or absence of lithocholate were equal, occurred at an MLV concentration of 3.77 nmol lipid/μl. This MLV concentration corresponded to a lithocholate/lipid molar ratio of 0.016, thus indicating that lithocholate-induced free calcim uptake was observed only at lithocholate/lipid molar ratios greater than 0.016.

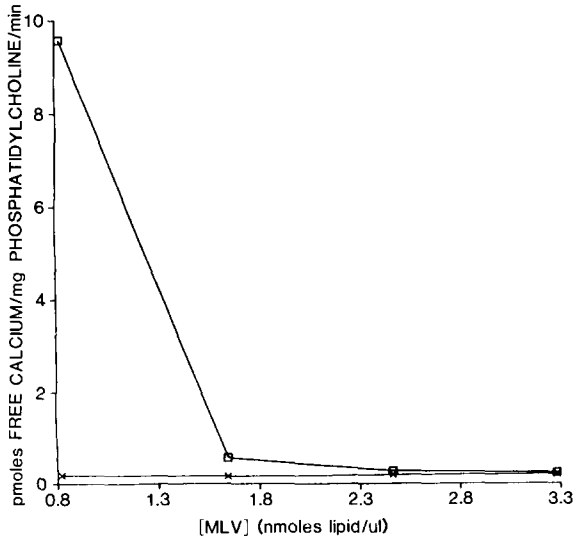

Fig. 7. Bile salt-induced free calcium accumulation by MLVs as a function of MLV concentration. The incubation medium was supplemented with 1 mM $CaCl_2$ and 0 (×) or 60 μM (□) lithocholate. Each point indicates the mean initial rate of free calcium change.

Effect of bile salts on monovalent cation accumulation

The specificity of taurodeoxycholate for calcium accumulation was examined in SUVs in two ways. In the first set of experiments, replacement of all calcium ion with either sodium or rubidium (employing ^{22}Na or ^{86}Rb, respectively) resulted in no significant taurodeoxycholate-dependent uptake of monovalent cations by SUVs. ^{22}Na and ^{86}Rb uptakes in the absence of taurodeoxycholate were 0.075 ± 0.025 and 0.150 ± 0.050 nmol/mg phospholipid per min, respectively. In the presence of taurodeoxycholate (750 μM), ^{22}Na and ^{86}Rb uptakes were 0.088 ± 0.013 and 0.175 ± 0.038 nmol/mg phospholipid per min, respectively. By comparison, ^{45}Ca uptakes in the absence and presence of taurodeoxycholate were 1.013 ± 0.050 and 2.700 ± 0.213 nmol/mg phospholipid per min, respectively. Under the second set of conditions rubidium and calcium were present together in the incubating medium, and the results were the same as above. Radiolabelled calcium accumulation was stimulated 2.5-fold by taurodeoxycholate, but identical conditions had no effect on ^{86}Rb accumulation. Collectively, these results suggested that taurodeoxycholate-induced permeability changes in SUVs were specific for calcium cation.

Electrochemical versus proton gradients

To delineate the forces affecting transport, the ionophores valinomycin and nigericin were employed with both SUVs and MLVs to determine whether bile salt-mediated calcium uptake was influenced by membrane potential or pH gradients. In the absence of taurodeoxycholate, the initial rate of calcium uptake by SUVs (1.03 ± 0.10 nmol calcium/mg phosholipid per min) was unchanged by the presence of valinomycin (25 nM) or nigericin (25 nM) (1.08 ± 0.13 or 0.95 ± 0.15 nmol/mg phospholipid per min, respectively). In the presence of taurodeoxycholate (250 μM), the

initial rate of calcium uptake (1.78 ± 0.15 nmol/mg phospholipid per min) was also unaffected by valinomycin (1.75 ± 0.10 nmol/mg phospholipid per min). However, nigericin increased the initial rate of calcium uptake to 2.50 ± 0.15 nmol/mg phospholipid per min in the presence of taurodeoxycholate – an increase of 40%.

Comparable experiments with MLVs and lithocholate yielded qualitatively similar results. Free calcium uptake by MLVs in the absence of bile salt (0.24 ± 0.03 pmol calcium/mg phosphatidylcholine per min) was unaffected by the presence of valinomycin (20 nM) (0.20 ± 0.01 pmol/mg phosphatidylcholine per min) and reduced by exposure to nigericin (20 nM) (0.13 ± 0.02 pmol/mg phosphatidylcholine per min). By contrast, uptake in the presence of lithocholate (60 μM) and nigericin (1.86 ± 0.67 pmol/mg phosphatidylcholine per min) was significantly greater by paired analysis ($P < 0.05$) than uptake in the presence of either lithocholate alone (1.08 ± 0.35 pmol/mg phosphatidylcholine per min) or lithocholate and valinomycin (1.46 ± 0.40 pmol/mg phosphatidylcholine per min) which were not significantly different from one another.

Discussion

Free calcium plays a key role in the promotion and regulation of multiple cellular processes. Because of the pivotal role of calcium in the maintenance of cellular well-being and initiation of cytopathic events, considerable interest has been generated in agents capable of translocating calcium across plasma membranes [20]. In this regard, Weissmann and co-workers proposed and developed the concept of 'endogenous calcium ionophores' as naturally produced compounds which at micromolar concentrations mediate the transfer of calcium across biological membranes [9,10].

Studies in our laboratory of the calcium-binding properties of certain of the cholestatic bile salts, and studies in other laboratories of the stimulatory activity of bile salts upon secretory cells, suggested that bile salts had an effect upon the calcium permeability of membranes [2–4]. In those earlier studies we looked at the effect of bile salts upon calcium permeability of erythrocyte membranes [4]. The presence of bile salts stimulated radiolabelled calcium uptake 4–25-fold, the magnitude of which was partly related to the lipid solubility (hydrophobicity) of bile salts. Similar investigations of radiolabelled calcium efflux from hepatocytes and calcium influx/efflux from jejunal brush border vesicles have also demonstrated the parallel relationship between ionophore activity and bile salt hydrophobicity [3,7].

In spite of the evidence, though, that bile salts induce calcium uptake, it still remains unsettled whether the observed calcium translocation is a nonspecific event resulting from generalized membrane leakiness and altered membrane pump activity, or whether it results from a more specific transport process. In the case of bile salts, the first possibility is particularly attractive because of the well-known capacity of bile salts for solubilizing certain membrane components without fully disrupting membrane integrity [5,21]. Another perceived difficulty with interpretation of the above investigation is that the observed calcium uptakes, as measured by radiolabelled calcium assays, might not represent translocated calcium at all, but rather bile salt-bound calcium in membrane or intracellular speces. Hence, to further investigation of bile salt-mediated calcium uptake in light of the above uncertainties, the present experiments were conducted employing two different systems for assaying calcium uptake by artificial vesicles. The first system employed a method for assaying radiolabelled calcium in SUVs. The advantages of this system were several-fold: (i) SUVs had no metabolic activities or other membrane transport processes which could interfere with the assay or which could be directly inhibited by bile salts; (ii) the internal composition of SUVs could be controlled in order to optimize conditions favoring calcium transport; and (iii) the uptake of monovalent as well as divalent solutes could be readily assayed using standard SUV preparations and analytical techniques which were well-established in our laboratory [12].

SUVs were prepared in salts of citric acid for two reasons. First, citric acid at pH 7.4 is present predominantly as the trivalent anionic citrate species. The relatively high charge associated with its hydrophilic nature rendered citrate virtually impermeable to phospholipid bilayers. By contrast, the ionic gradients of sodium or potassium

salts of permeant anions such as thiocyanate, nitrate, or chloride would have dissipated fairly rapidly after dilution or replacement of the external salts, Substitution of citrate as the major intravesicular anion permitted the maintenance of both monovalent cationic and proton gradients at constant levels for periods of at least 1 h [8]. Second, citric acid is a known chelator of calcium ions in the submillimolar range ($pK_d = 3.6$). The presence of citric acid in the intravesicular space facilitated calcium accumulation by maintaining a low internal free calcium concentration, thus maximizing the chemical gradient for calcium across the vesicular membrane. Indeed, such a phenomenon was observed in studies of the reconstituted Ca^{2+}-ATPase which exhibited maximal rates of transport only when a calcium buffer (in those experiments, oxalate) was included in the vesicle interior [22].

The second system for assaying bile salt-induced calcium uptake employed MLVs loaded with a free calcium indicator, arsenazo III. Large unilamellar vesicles were not used because of their reported transformations to MLVs following introduction of millimolar concentrations of calcium [6]. MLVs loaded with arsenazo III offered most of the advantages outlined above for SUVs in addition to the following benefits: (i) the substitution of MLVs for SUVs simplified vesicle preparation, increased intravesicular aqueous volumes, reduced the high surface to volume ratio associated with SUVs, and improved encapsulation efficiency, thus promoting entrapment of arsenazo III; (ii) the use of purified lipids provided greater control over vesicle composition and minimized the presence of contaminating protein or calcium; and (iii) the substitution of a free calcium indicator for radiolabelled calcium eliminated possible contributions of lipid-, bile salt-, citrate-, or protein-bound calcium from measurements.

Comparison of MLVs prepared in our laboratory to those reported by Weissmann and others suggest that the preparations were comparable with respect to lipid composition, captured volume, and entrapment efficiency [23]. The empirically determined values for $\Delta\epsilon$ and K_d of the arsenazo III-Ca complex were also in close agreement with reported values [18]. It is conceivable that the observed bile salt-induced free calcium changes were not the result of changes in intravesicular free calcium concentration, but rather, changes in intravesicular pH. However, this possibility seems unlikely for several reasons: the intravesicular solution was well-buffered; a decline in pH, as might have occurred in the presence of bile salts, would more likely have decreased rather than increased $\Delta A_{656-700}$, thus decreasing the apparent free calcium concentration [18]; $\Delta\epsilon$ and K_d values were unaffected by bile salt presence in MLV-free solutions; and there was no observed change in $\Delta A_{656-700}$ values following the addition of bile salt in the absence of calcium to MLVs loaded with arsenazo III. Although it is unlikely that changes in intravesicular pH significantly influenced $\Delta A_{656-700}$ values during most experiments for the above reasons, it does remain possible that a nigericin-induced drop in pH may have accounted for the lower calcium uptake by MLVs observed in the presence of nigericin and absence of bile salt.

To assess the occurrence of membrane solubilization during bile salt-induced calcium accumulation, the release of nonpermeant solutes from SUVs and MLVs was measured at bile salt concentrations greater than and less than the published critical micellar concentration of taurodeoxycholate. Because the critical micellar concentration of taurodeoxycholate is about 1 mM, it was not surprising that retained solute was less than 60% as concentrations approached or exceeded this value. Based on those results, guidelines for maximal bile salt concentrations were developed that would minimize membrane solubilization. Although we believe that adherence to these concentration restrictions minimized vesicle disruption in subsequent experiments, it does not necessarily follow, however, that all membrane solubilization of a less disruptive nature was eliminated.

Calcium accumulation, as indicated by the net flux of radiolabelled calcium in SUVs or by the net change of intravesicular free calcium content in MLVs, was significantly promoted by bile salts. Taurodeoxycholate was chosen for comparison with taurocholate in SUVs because of the potent calcium uptake-promoting activity of taurodeoxycholate in erythrocytes [4] and the favorable aqueous solubility of both bile salts at millimolar

concentrations. Comparison of these bile salts with other unconjugated bile salts in MLVs, however, revealed that lithocholate and 3β-hydroxy-5-cholenoate were more potent bile salts for inducing calcium uptake at micromolar concentrations. Overall, calcium accumulation was directly related to the hydrophobicity of bile salts as determined by the elution profiles of unconjugated bile salts and their taurine conjugates by reverse phase HPLC [24]. Although 3β-hydroxy-5-cholenoate was approx. 9-times more potent than lithocholate in these experiments, the latter was chosen for further investigation because of more favorable solubility characteristics, better characterization of physicochemical properties, and greater significance to physiologic processes.

SUVs exhibited a relatively large endogenous 'leak' to, or nonspecific binding of calcium in the presence and absence of bile salts. It is not clear whether this represented a small population of leaky vesicles or whether it represented nonspecific calcium binding to the membrane surface. This was not unexpected, however, due to the magnitude of the electrochemical gradient into the vesicle engendered by the presence of a calcium chelator in the interior of the vesicle. Even though SUV and MLV results from the present studies were not directly comparable because of multiple methodologic differences between the two protocols, the difference between calcium uptake by SUVs and MLVs that was 1000-fold greater in SUVs is consistent with the above suggestion that a portion of measured calcium uptake by SUVs was bound calcium. Regarding the presence of other possible chelators in the system, studies of bile salt-induced calcium efflux from Ca^{2+}-ATPase vesicles have indicated increased entry of calcium-bile salt complexes and persistent calcium binding by intravesicular bile salts [8].

Vesicle concentration profoundly influenced bile salt-mediated free calcium accumulation in MLVs. Specifically, the reciprocal effects of lithocholate and MLV concentrations on calcium uptake were consistent with a direct influence of lithocholate/lipid molar ratios. By comparison with other proposed endogenous calcium ionophores, the minimally effective ionophore/lipid molar ratio of lithocholate was similar to that reported for phosphatidate [10]. On the other hand, the minimal effective ionophore/lipid molar ratios of endogenous ionophores are 10000- and 1000-fold greater than those reported for ionomycin and A23187, respectively [9]. These differences in ionophore/lipid molar ratios between endogenous ionophores and ionomycin or A23187 are consistent with ionomycin and A23187 activity at nanomolar concentrations which we have observed in both SUVs and MLVs.

The mechanism of calcium transport by bile salts remains undefined. While other mechanisms are conceivable, we considered three pathways by which bile salts might influence membrane permeabilities. The first mechanism proposes that permeability changes relate to generalized disruption of membrane integrity. This mechanism becomes important as bile salt concentrations approach or exceed the critical concentration at which mixed micellization of membrane components occurs. Release of either radiolabelled choline by SUVs or arsenazo III by MLVs provided evidence of such detergent action at taurodeoxycholate concentration approximating 1 mM. The second mechanism proposes that bile salts induce channel formation by the development of suitably structured bile salt complexes in membrane. Channels might exert cation selectivity by maintaining a specific pore size and charge orientation. In the third mechanism bile salts may function as electrically neutral, lipid-soluble carriers which release calcium in exchange for another cation. Such is the mechanism whereby calcium ionophores A23187 and ionomycin exchange calcium ions for protons across membranes.

To compare the influence of $\Delta\psi$ versus ΔpH upon rate of calcium accumulation, the ionophores valinomycin and nigericin were employed to generate electrochemical or proton gradients. Valinomycin is a potassium-specific conductive ionophore which in these vesicle systems generated a $\Delta\psi$, the magnitude of which was dependent upon the potassium ion concentration gradient [12]. Introduction of a potassium conductance with valinomycin permitted the generation of a membrane potential which was inside-negative. On the other hand, addition of a potassium/proton exchanger, nigericin, permitted the generation of a pH gradient as a consequence of ionophore-mediated potassium efflux that was coupled to proton

uptake. By these experiments, bile salt-mediated calcium uptake was stimulated by electrically neutral, potassium/proton exchange. This comparison of the effect of membrane potential and proton gradients upon rate of calcium uptake did not define the mechanism by which bile salts promoted calcium transport in these investigations. However, based on these results the possibility is raised that bile salts transport calcium as a neutral complex, release calcium at the internal membrane surface in exchange for protons, shuttle back across the membrane as a neutral protonated complex, and exchange protons for calcium at the external membrane surface. Based on the current studies, the possibility of the bile salt-induced uptake of divalent cations other than calcium is not excluded.

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