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The mode of action of primary bile salts on human platelets

Young-Ji Shiao [a], Jia-Chyuan Chen [b], Chuen-Neu Wang [a] and Cheng-Teh Wang [a]

[a] *Institute of Life Science, National Tsing Hua University, Hsinchu (Taiwan) and*
[b] *Department of Zoology, National Taiwan University, Taipei (Taiwan)*

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Cholate and its conjugated amide derivatives glycocholate and taurocholate solubilized human platelets differently as studied by the observations on: (1) the change in optical absorbance of platelet suspension, (2) marker leakiness and (3) component solubility. Cholate ruptures the membrane in an all-or-none process, while both conjugated derivatives shed off both proteins and lipids. The shed lipids formed vesicles and could be separated from the proteins. The conjugated salts gradually chop off the cell membrane into pieces causing the cells to become small spheres (1.5 μm in diameter) as revealed by scanning electron microscopy, which also revealed that morphological change of platelet in these bile salts depended on both concentration and incubation period. Platelets at the prelytic-stage concentration of these three salts deformed initially to spiculate disc and finally to a stretched-out flat form. Also, in the prelytic stage of these bile salts, platelets showed inhibited responses to thrombin which did not happen to platelets in deoxycholate (Shiao et al. (1989) Biochim. Biophys. Acta 980, 56–68.).

Introduction

The primary bile salts are cholate ($3\alpha,7\alpha,12\alpha$-trihydroxy-5β-cholan-24-oic acid) and its conjugated amide derivatives glycocholate and taurocholate (Ref. 1, and references therein). These bile salts are made in liver and secreted at the combined concentration of 20 mM in hepatic bile. The conjugate derivatives compose about half the bile salt pool. The salts form mixed micelles with lipids to facilitate the digestion and the absorption of dietary lipids. Most of the molecules in the bile salt pool are reabsorbed from the intestines, wherein some may either undergo partial dehydroxylation to secondary bile salts or become deconjugated. In their circulation back into liver, these products are often rehydroxylated and reconjugated. Bile salts are detergents. Cholate and the secondary bile salt deoxycholate ($3\alpha,12\alpha$-dihydroxycholan-24-oic acid) are extensively used to solubilize membrane proteins [2,3]. Glycocholate and taurocholate are considered to be mild detergents and were found to cause shedding of membrane glycoproteins from bile canalicular cells, erythrocytes and lymphocytes [4–6].

To solubilize a membrane, a detergent infiltrates the membrane bilayer and converts it into the mixed micelles of detergent and membrane constituents. The solubilization occurs in three stages depending on the detergent concentration [7,8]. The prelytic stage represents the concentration at which the detergent enters membrane without rupturing the bilayer structure. The lytic stage begins once the concentration in membrane reached saturation. When all membrane constituents become mixed micelles, this marks the onset of complete-cell-solubilization stage. The chemical nature of a detergent determines its solubilizing ability. Its critical micellar concentration (CMC) is one of the important factors reflecting detergent–protein interaction. The CMC values of various bile salts are in the mM range with the order of the conjugated cholate derivatives $\geqslant$ cholate > deoxycholate [9–13]. The bile salt molecules have a planar hydrophobic moiety, and are considered to incorporate into membrane in oligomeric form [3].

Recently, we have reported that detergents with different physical properties affect human platelets differently [7]. It showed that deoxycholate at the concentration of platelet-prelytic stage inhibits platelet responses to both collagen and ADP-fibrinogen but not to thrombin. In this study, we reported the differential

Correspondence to: C.-T. Wang, Institute of Life Science, National Tsing Hua University, Hsinchu, Taiwan 30043, Republic of China.
Abbreviations: CMC, critical micellar concentration; PSC, platelet saturation concentration; MCPLC, minimum complete platelet-lysis concentration; S_{50}, the detergent concentration required to solubilized 50% of an indicated component.

solubilizations of human platelet by cholate, glycocholate and taurocholate. The solubilization was investigated by analyzing the change in optical absorbance of platelet suspension, marker leakiness, and solubility of membrane components. The result indicated that the solubilization of platelet by these detergents occurred in three stages. The platelet saturation concentration (PSC) and the minimum complete-platelet-lysis concentration (MCPLC) were estimated. The former is the detergent concentration at which the conversion of platelet membrane to mixed micelles begins, and the latter is the minimum detergent concentration required for all of membrane components to become mixed micelles. In the prelytic stage, further studies were performed to observe the effects of these trihydroxy bile salts on platelet morphology by scanning electron microscopy and on the agonist-stimulated response. The results showed that cholate and its derivatives affected platelet morphology differently, but they all inhibited the thrombin response.

Materials and Methods

Materials

Chemicals, organic solvents, sodium deoxycholate, bis(trimethylsilyl)trifluoroacetamide, EDTA (Titriplex II) and thin-layer chromatographic plate (TLC plate silica gel 60) were purchased from E. Merck (Darmstadt, Germany). All organic solvents were redistilled before use. The following reagents were obtained from Sigma (St. Louis, MO, USA): glycocholate, taurocholate, chenodeoxycholate, adenosine diphosphate (platelet aggregation agent), fibrinogen (human), phorbol 12-myristate 13-acetate, bovine thrombin, collagen (Type III from calf skin) and NADH. Cholate was from Serva (Heidelberg, Germany) and Sepharose 2B was from Pharmacia (Piscataway, NJ, USA). Fura-2/AM was from Boehringer Mannheim (Mannheim, Germany). SDS-PAGE molecular weight standards were purchased from Bio-Rad (Richmond, CA, USA).

According to the manufacturers, these bile salts were all $\geqslant 97\%$ pure. Each authentic sample (200 μg per application) gave a single spot as checked by silica gel 60 thin-layer chromatography and visualized by iodine. The solvent system to separate conjugated cholate derivatives was chloroform/methanol/glacial acetic acid/water (15:5:1:1, by volume) [14], while the system to separate unconjugated bile salts was benzene/dioxane/glacial acetic acid (20:10:2, by volume) [15].

Preparation of gel-filtered platelets

The preparation of gel-filtered human platelets has been detailed previously [16,17]. It was carried out according to the method of Lages et al. [18]. Human blood was freshly drawn from healthy donors and anticoagulated with 10% (v/v) of 0.11 M sodium citrate. The buffer solution was modified calcium-free Hepes-Tyrode's buffer containing 0.1% dextrose and 5 mM Hepes (pH 7.4). The isolated platelets were preincubated at 37°C for 30 min prior to further studies [17].

Analyses of the changes in optical turbidity of platelet suspension

The optical turbidity of platelet suspension was observed in an aggrecorder (Daiichi, Model PA-3220, Kyoto, Japan). The optical absorbance at 650 nm of $3 \cdot 10^8$ cells/ml was 0.38, which was designated as total absorbance (T). Upon the addition of bile salt, the change in the absorbance of cell suspension was followed for 50 min. The maximun change in absorbance was designated as A. Hence, the % of change in absorbance was obtained from $((T-A)/T) \times 100\%$.

Estimation of the marker leakiness

Platelets ($3 \cdot 10^8$ cells/ml) were incubated with bile salts at various concentrations. After a certain period, aliquots were introduced with 2.5 mM EDTA and then centrifuged in a laboratory centrifuge (Sigma Model 202 MC, Germany) at $13\,500 \times g$ for 1 min. The supernatant (so-called the first supernatant) was analyzed for the content of various cellular markers. The activity of lactate dehydrogenase (cytosolic marker) was estimated according to the method of Bergmeyer and Bernt [19], and that of acid hydrolase (lysosomal marker) was measured by following the method of Dangelmaier and Holmsen [20]. The substrate of the latter enzyme was *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide. The bile salts, Triton X-100 and EDTA did not affect these markers. Hence, 100% of each marker was the value estimated in the supernatant of platelet treated with 0.1% Triton X-100.

Estimation of the component solubilization

In various bile salts, the studies on the time-course of the component solubilization were performed as described above. For estimating the protein and lipid solubilities, the obtained supernatant and pellet were both quantitated to calculate the percentage of protein and lipid solubility. The protein content was determined according to the method of Lowry et al. [21]. Lipid were extracted according to the method of Bligh and Dyer [22]. Phospholipid composition was analyzed by two-dimensional thin-layer chromatography as described previously [16], and quantitated by estimating the phosphorus content [23,24]. Cholesterol content was estimated by gas chromatography (Shimadzu, GC-9A, Japan) as described by Derks et al. [25]. The lipid derivatives of bis(trimethylsilyl)-trifluoroacetamide were separated at 240°C with a flow rate of 40 ml/min. The column (3×2 mm) was packed with 10% SP 2330

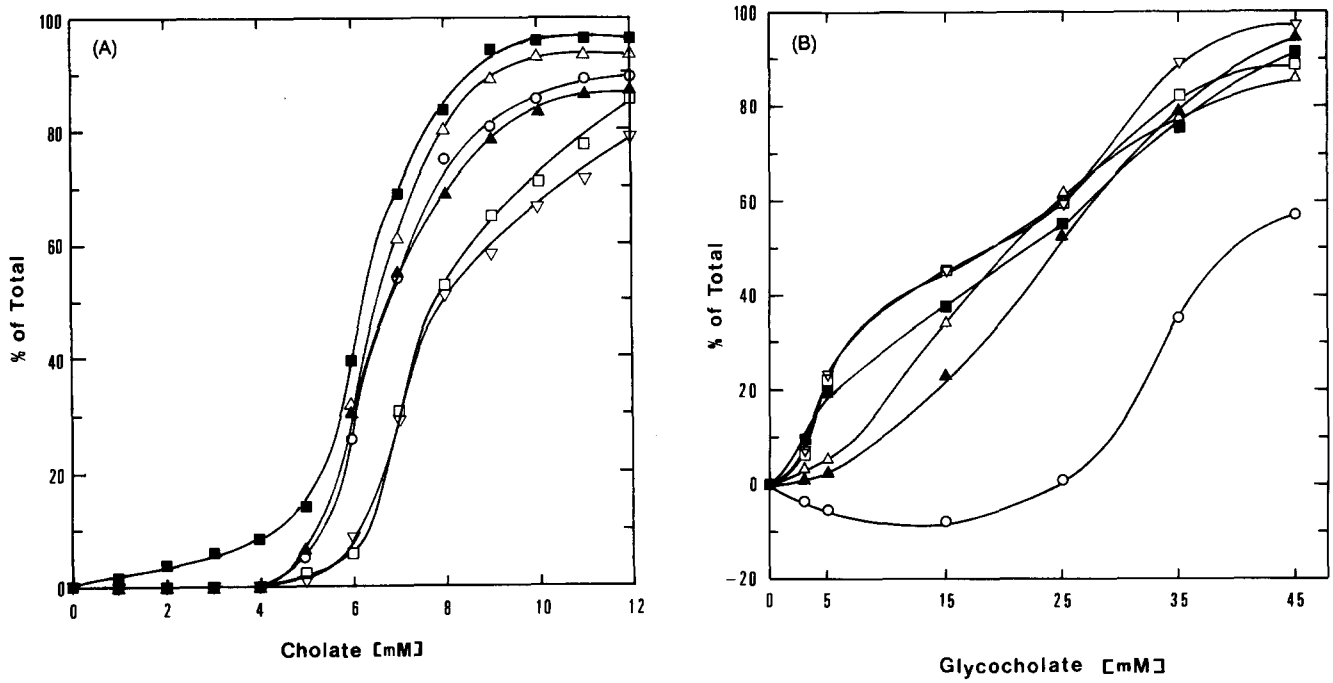


Fig. 1. Solubilization of human platelets by primary bile salts. Gel-filtered platelet ($3 \cdot 10^8$ platelets/ml) were incubated for 10 min with various concentrations of cholate (A), glycocholate (B) and taurocholate (C). Materials and Methods detail the estimations of the percentage of the change in absorbance at 650 nm of platelet suspension (○); the leakiness of lactate dehydrogenase (△); and acid hydrolase (▲); and the solubilities of phospholipid (□); protein (■); and cholesterol (▽). Data points were taken from an average of at least six separate experiments, the standard derivation of data points was within 15%.

on chromsorb WAW-DMCS 80/100 (Supelco, Bellefonte, PA, USA).

For the vesiculation studies, a second-step centrifugation was performed on the first supernatant, which was recentrifuged at 350 000 × g for 1 h in an ultracentrifuge (Beckman, Model TL100, USA). The second supernatant and pellet were re-estimated for the content of proteins and lipids as described above, while the pellet was also examined by negative-staining electron microscopy.

Proteins of these three fractions were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli [26]. The stacking and separating gel contained 3.7% and 11% of polyacrylamide, respectively. The second supernatant was dialyzed against water at 4°C overnight to remove detergent, and proteins were concentrated. Three volumes of each sample were vortex-mixed with one volume of the sample buffer containing 8% SDS, 20% 2-mercaptoethanol, 40% glycerol and 0.25 M Tris-HCl (pH 6.8). The mixture was boiled in a water bath for 3 min. 10 μg of each sample was loaded per well. After electrophoresis, gels were stained with silver stain [27].

Morphological studies by electron microscopy

Negative-staining electron microscopy was employed to examine the pellet obtained from the above described two-step centrifugation. The pellet was resuspended in 0.1 ml of Hepes-Tyrode's buffer. A drop of sample was put on a carbon-coated Formvar grid (300 mesh). After negative staining with 2% phosphotungstic acid for 30 s, the sample was examined in a Hitachi H-600 transmission electron microscope at 75 kV.

The sample preparation for scanning electron microscopic study was detailed in a previous study [17]. In brief, the bile salt-treated platelets were added with 5 vol. of ice-cold 2.5% glutaraldehyde in the modified calcium-free Hepes-Tyrode's buffer (pH 7.4). After storage in ice for 1 h, the prefixed sample was then postfixed with 1% OsO_4 and dehydrated. The sample was dried in a critical-point dryer (Hitachi, Model HCP-2, Japan) in CO_2, and then plated with gold in an ion coater (Eiko, Engineering, Model IB-2, Japan). The plated sample was observed under a Hitachi S-520 scanning electron microscope at 20 kV.

Other analyses

Fura-2/AM was employed as a probe in the estimation of the change in $[Ca^{2+}]_i$ of platelet in various prelytic concentration of bile salts. The experiment was performed according to the method of Pollock et al. [28], with some modification as described by Wang et al. [29].

The study of agonist-stimulated response was performed in an aggrecorder (Daiichi, Model PA 3220, Kyoto, Japan). The agonists were thrombin (0.1

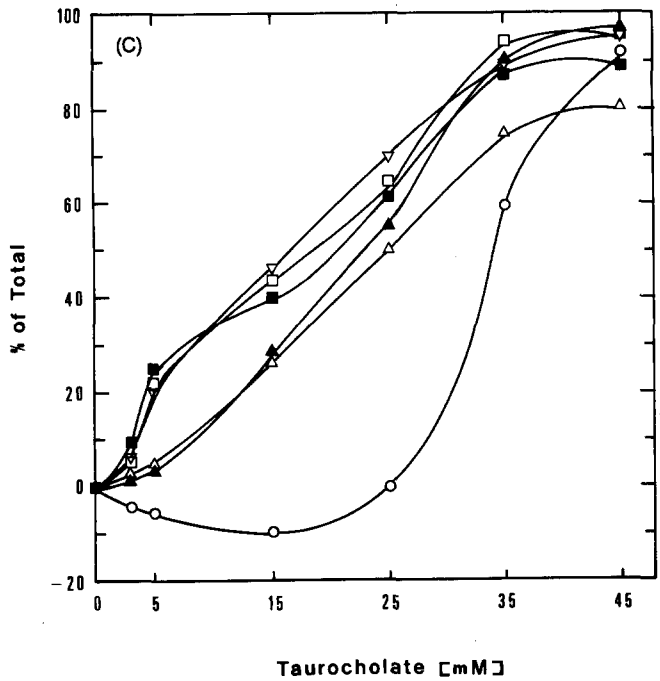


Fig. 1. (continued).

unit/ml), collagen (100 μg/ml), ADP (20 μM) plus fibrinogen (100 μg/ml), and phorbol 12-myristate 13-acetate (10 ng/ml).

Results

Solubilization of human platelet by cholate, glycocholate and taurocholate

Fig. 1 depicts the concentration-dependent solubilization of gel-filtered platelets by cholate, glycocholate and taurocholate. The solubilization was analyzed by estimating the percentage of: (1) the change in optical absorbance of platelet suspension; (2) marker leakiness; (3) solubility of phospholipids; cholesterol; and proteins. The markers were lactate dehydrogenase and acid hydrolase. These data were taken from the observation by incubating platelets with these bile salts for 10 min, which was the incubation period necessary for the absorbance curves to reach plateau at various concentrations producing the change in optical turbidity. So it was designated as the lysis time in Table I. The table also shows the estimated S_{50}, i.e., the concentration of a detergent required to solubilize 50% of each component indicated.

Platelets in cholate showed the comparable sigmoid curves of the three analyses. The protein solubilization, marker leakiness and the change in absorbance occurred at a lower concentration than those of lipid solubilization. It means that cholate might rupture platelet membrane in an all-or-none process.

The solubilization of platelets in both glycocholate and taurocholate appeared to be similar (Figs. 1B and 1C). In contrast to the cholate-solubilization of platelets, the conjugated derivatives first solubilized both proteins and lipids (20% of each at 5 mM of the detergents), and then followed by the leakiness of lactate dehydrogenase (20% of leakiness at 10 mM). The phospholipid composition in the shed fraction was similar to that in whole cell (data not shown). It may mean that the membrane shedding was from both leaflets of membrane bilayer. The changes in the absorbance of cell suspension increased up to 15 mM,

TABLE I

The physical properties of various bile salts in the solubilization of human platelet

Physical property	Trihydroxy bile salts			Dihydroxy bile salts	
	cholate	glyco-cholate	tauro-cholate	deoxy-cholate [a]	cheno-deoxy-cholate
PSC (mM) [b]	4.8	5.0	5.0	0.97	0.8
CMC (mM) [c]	3.0	6.1–13.0	2.7–14.0	0.91	1.5–2.3
PSC/CMC	1.6	0.8– 0.4	1.9– 0.4	1.07	0.5–0.4
Lysis time [d] (min)	10	10	10	20	10
MCPLC (mM) [b]	8	45	45	2.60	1.4
MCPLC/PSC	1.7	9	9	2.68	1.8
S_{50} (mM) [e]					
Protein	6.4	24	22	1.20	0.8
Phospholipid	7.5	18	18	1.84	0.9
Cholesterol	8.0	18	18	2.08	0.9

[a] The data were taken from our previous work [6].

[b] PSC (platelet-saturation concentration) and MCPLC (minimum complete plateletlysis concentration) were determined from the concentration causing 5% and 80% of lactate dehydogenase leakiness, respectively.

[c] CMC (critical micellar concentration) was taken from the data of previous studies [8–12].

[d] Lysis time represents the required incubation period for the complete solubilization of platelets in various lytic concentrations.

[e] S_{50} is the detergent concentration required to solubilize 50% of each component indicated.

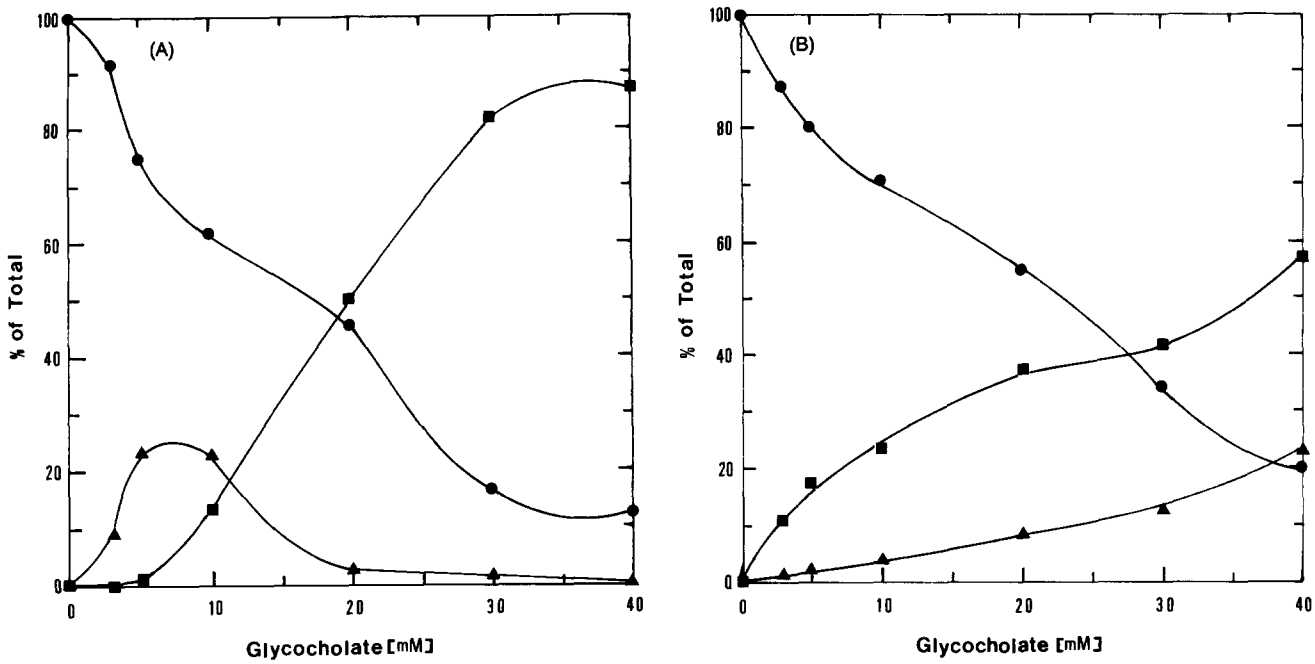


Fig. 2. The distributions of both phospholipids (A) and proteins (B) in the three fractions of glycocholate-treated platelets separated by a two-step centrifugation. Gel-filtered platelets were incubated with various concentrations of glycocholate for 10 min and centrifuged at a low speed centrifugation to obtain the first pellet (●) and the first supernatant which was centrifuged again at an high speed to obtain the second pellet (▲) and second supernatant (■). The estimations of both phospholipid and protein in each fraction were detailed in Materials and Methods. Data points were taken from an average of at least six separate experiments, the standard derivation of data points was within 15%.

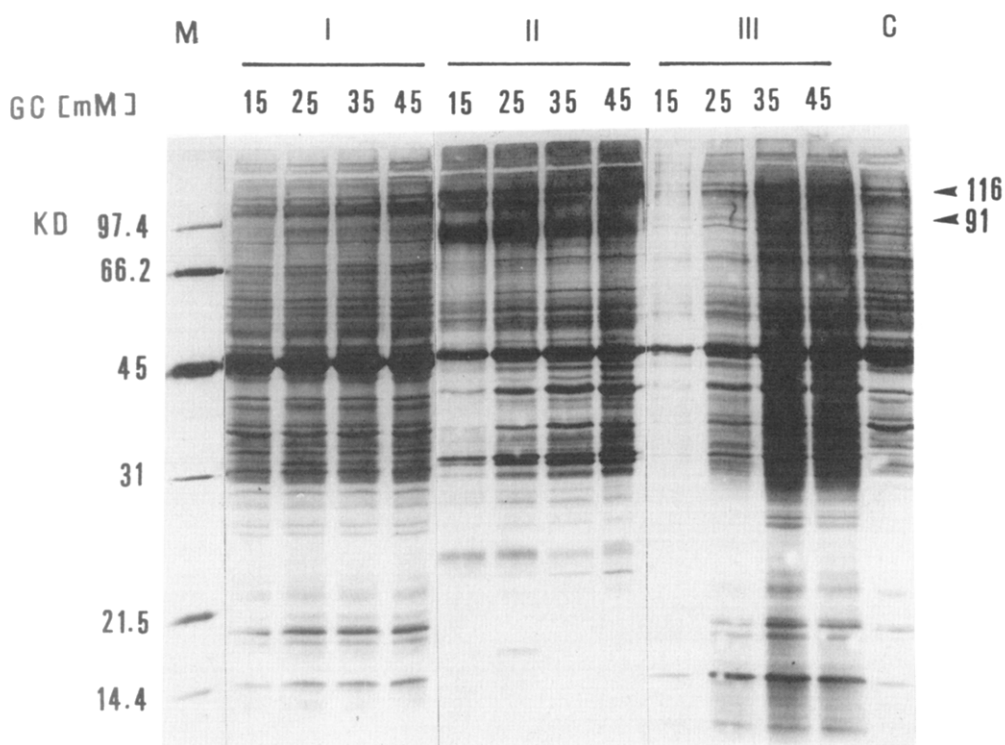
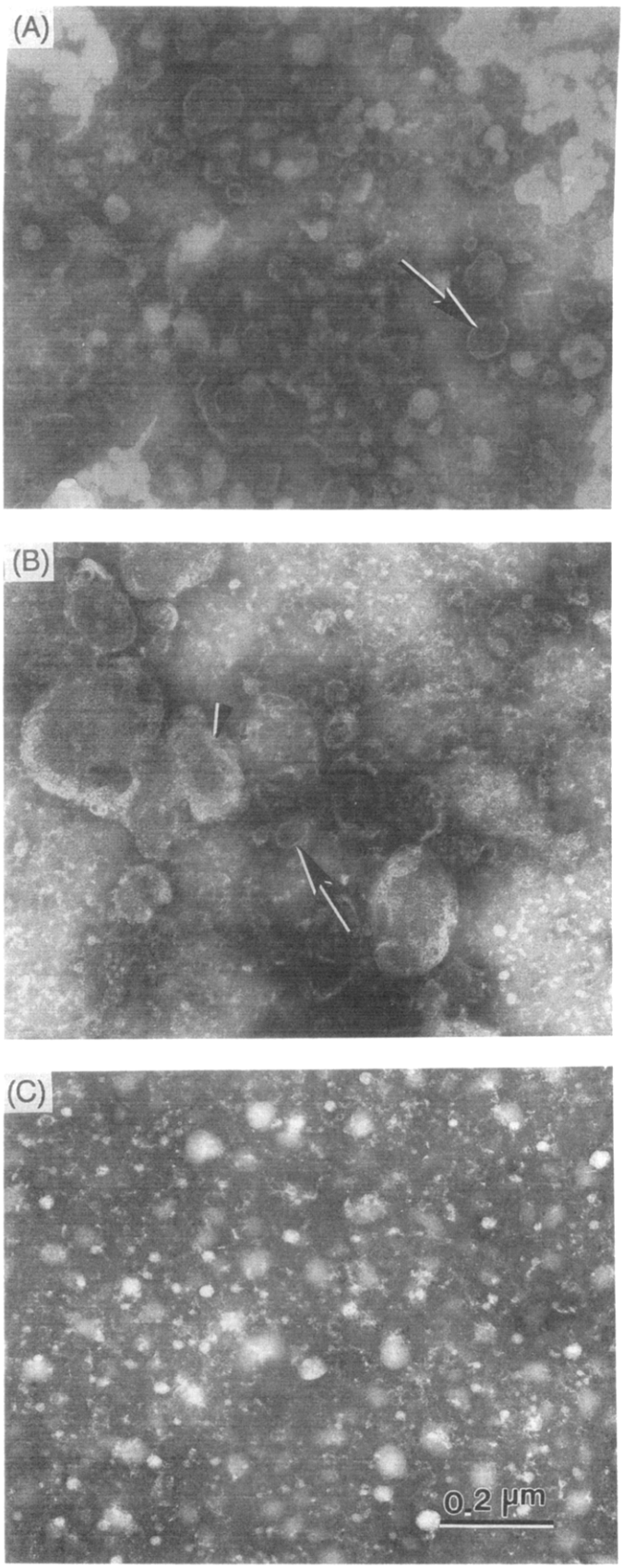


Fig. 3. SDS-PAGE of three fractions obtained from platelets treated by various concentrations of glycocholate (GC). Gel filtered platelets ($3 \cdot 10^8$ cells/ml) were incubated with various concentrations of glycocholate for 10 min. The samples were subjected to a two-step centrifugation. Proteins in first pellet (I), second pellet (II) and second supernatant (III) were separated by SDS-PAGE. After electrophoresis, the gels were stained by silver stain. Details of experiment are described in Materials and Methods. C, Control GFP; M, markers (97.4 kDa, rabbit muscle phosphorylase *b*; 66.2 kDa, bovine serum albumin; 45 kDa, hen egg white ovalbumin; 31 kDa, bovine carbonic anhydrase; 21.5 kDa, soybean trypsin inhibitor; 14.4 kDa, hen egg white lysozyme).



while those changes decreased at > 25 mM. It implies that the shed fraction contributed the light scattering activity in the suspension.

Two physical parameters, PSC and MCPLC, were estimated from the sigmoid curves of lactate dehydrogenase leakiness, in which the bile salt concentrations caused 5% and 80% of leakiness were designated as PSC and MCPLC, respectively. These data are summarized in Table I. The PSC of each bile salt might reflect its membrane saturation concentration in the platelet. Although various studies found different CMC values of both glycocholate and taurocholate [9–13], the ratios of PSC/CMC of these two cholate derivatives were in the same range. The ratio of MCPLC/PSC was 1.7 for cholate and 9 for both glycocholate and taurocholate, implying the different processes between cholate and its conjugated derivatives in rupturing platelet membrane.

Since the conjugated cholate derivatives shed off both proteins and lipids, a two-step centrifugation was carried out to see how these membrane components were solubilized. Platelets were treated with various concentrations of glycocholate and then subjected to a low-speed centrifugation to obtain the first pellet (so-called remnant platelets). The first supernatant was then centrifuged at a high speed to obtain the second pellet (so-called shed components), and the second supernatant was mixed micelles. The distribution of proteins and phospholipids in these three fractions were analyzed (Fig. 2). SDS-PAGE revealed the protein profiles of these three fractions (Fig. 3). The shed components were rich in proteins with the apparent molecular masses of 116 and 91 kDa, while the mixed micelles were rich in proteins of < 20 kDa. The second pellet was also observed by negative-staining electron microscopy (Fig. 4). The second pellet of platelets treated at 3 mM appeared to be only phospholipids (Fig. 2A), which were in vesicular forms with 40–100 nm in diameter (Fig. 4A), and solubilized proteins were in the mixed-micelle fraction (Fig. 2B). The vesicles became larger (150 nm in diameter) at 5 mM (Fig. 4B). The second pellet of platelets at > 30 mM glycocholate consisted only of protein aggregates with a diameter of about 15 nm (Figs. 2B and 4C), and lipids were in the mixed-micelle fraction (Fig. 2A). Hence, glycocholate of a low concentration shed off platelet lipids as vesicles, and solubilized proteins into mixed micelles.

Fig. 4. The negative-staining electron micrographs of the second pellets obtained from a two-step centrifugation of platelets at 3 mM (A), 5 mM (B) and 45 mM (C) glycocholate for 10 min (×80000). Arrow and arrow head indicate the small vesicles (40–100 nm in diameter) and the large vesicles (150 nm in diameter), respectively. Details of the experiments were given in Materials and Methods.

Morphological change of platelets in cholate, glycocholate and taurocholate

The marker leakiness studies indicated that more than 90% of the population were intact platelets at the prelytic-stage concentrations of these primary bile salts (Fig. 1). Interestingly, the cytosolic free Ca^{2+} concentrations of these detergent-treated platelets were similar to the control (data not shown). Hence, scanning electron microscopy was employed to investigate how platelet morphology was affected by these detergents (Figs. 5–7). The morphological changes appeared to be different between platelets in cholate (Fig. 5) and in the conjugated derivatives (Fig. 6), while the changes of platelets in glycocholate and in taurocholate were similar (data not shown). In various prelytic concentrations, the morphological changes of platelets in these detergents depended on both concentration and incubation period (Figs. 5–7).

Platelets in cholate changed morphology to two cell types (Fig. 5). The first type is the spiculate sphere, and the second type is the stretched-out flat shape (Figs. 5B–5D). The population distribution of these two cell types was both dose- and time-dependent (Figs. 7A, 7C and 7E). The spiculate spherical cells (first type) were more numerous at either a low concentration or at a short incubation period, while the stretched-out flat cells (second type) increased at either a high concentration or a prolonged incubation. Also, after a prolonged incubation, the rim of the second type cell was more extended (compare Figs. 5C and 7E). Cholate

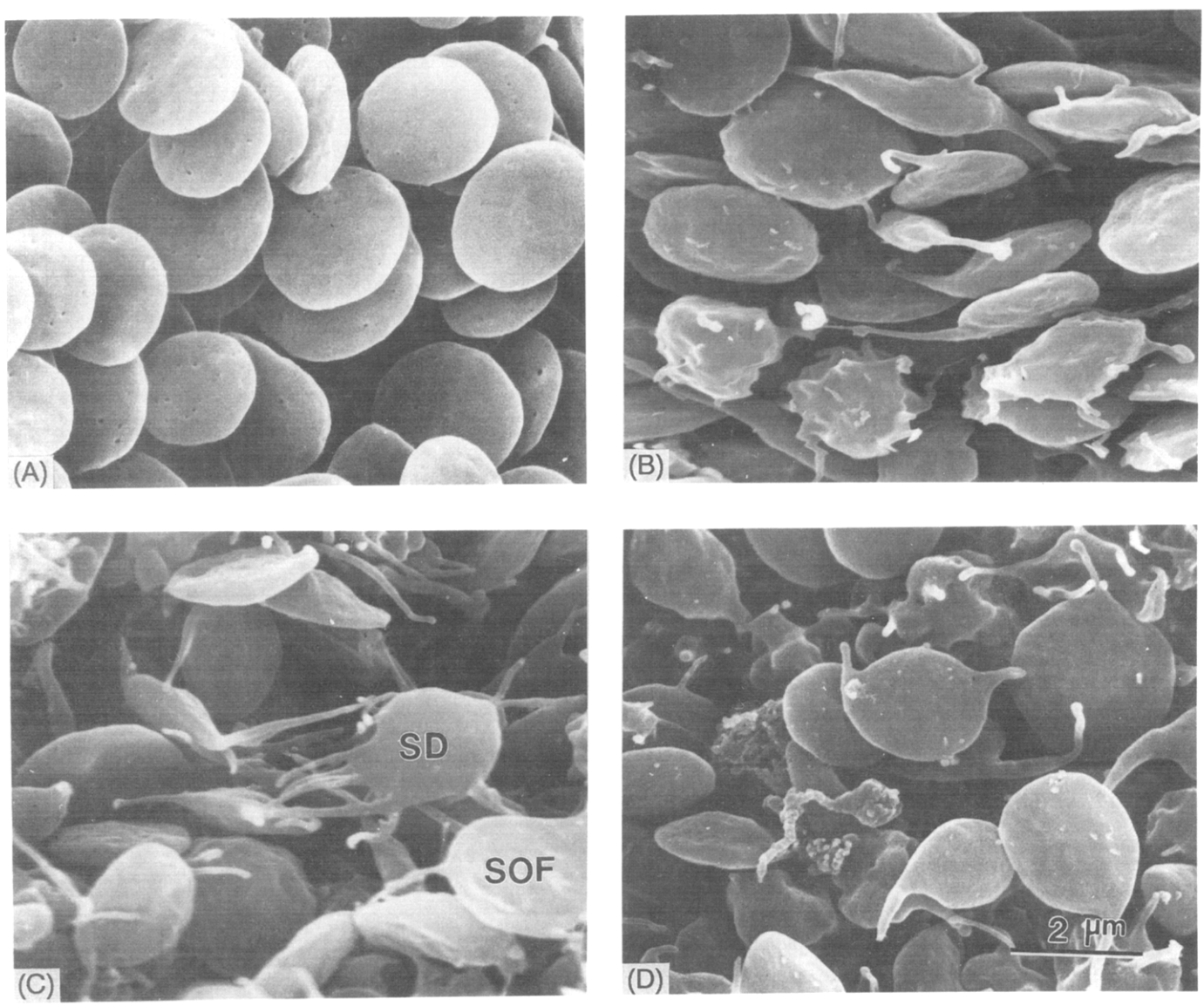


Fig. 5. Scanning electron micrographs of gel-filtered platelets in various concentrations of cholate (×10000). The gel-filtered platelets were preincubated at 37°C for 30 min (A), and then incubated for another 10 min with cholate in various prelytic concentrations of 3 mM (B); and 5 mM (C); and in lytic concentration of 9 mM (D). The abbreviations in the figure are SD, spiculate discoid cell, and SOF, stretched-out flat cell. Details of the experiments were given in Materials and Methods.

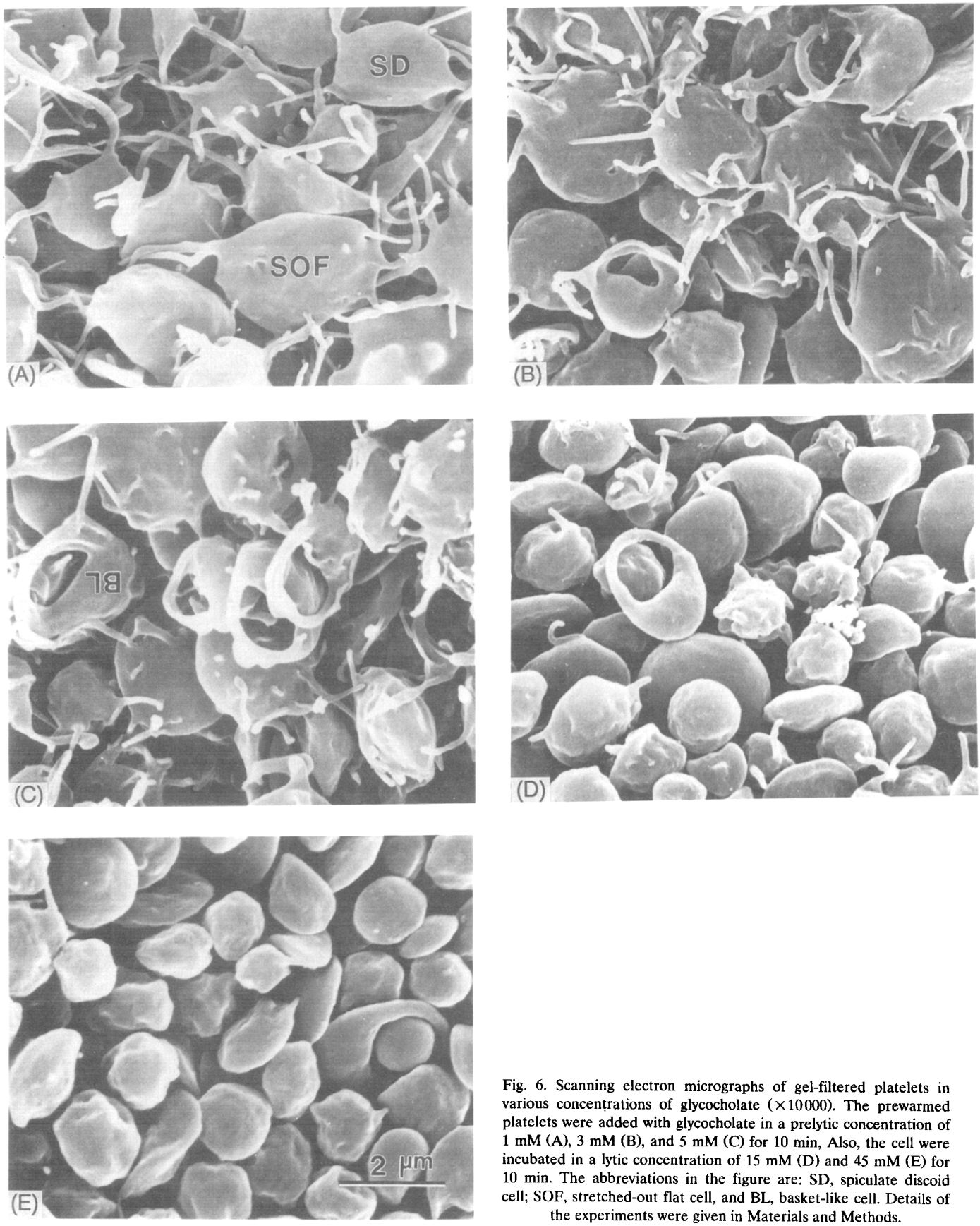


Fig. 6. Scanning electron micrographs of gel-filtered platelets in various concentrations of glycocholate (×10000). The prewarmed platelets were added with glycocholate in a prelytic concentration of 1 mM (A), 3 mM (B), and 5 mM (C) for 10 min, Also, the cell were incubated in a lytic concentration of 15 mM (D) and 45 mM (E) for 10 min. The abbreviations in the figure are: SD, spiculate discoid cell; SOF, stretched-out flat cell, and BL, basket-like cell. Details of the experiments were given in Materials and Methods.

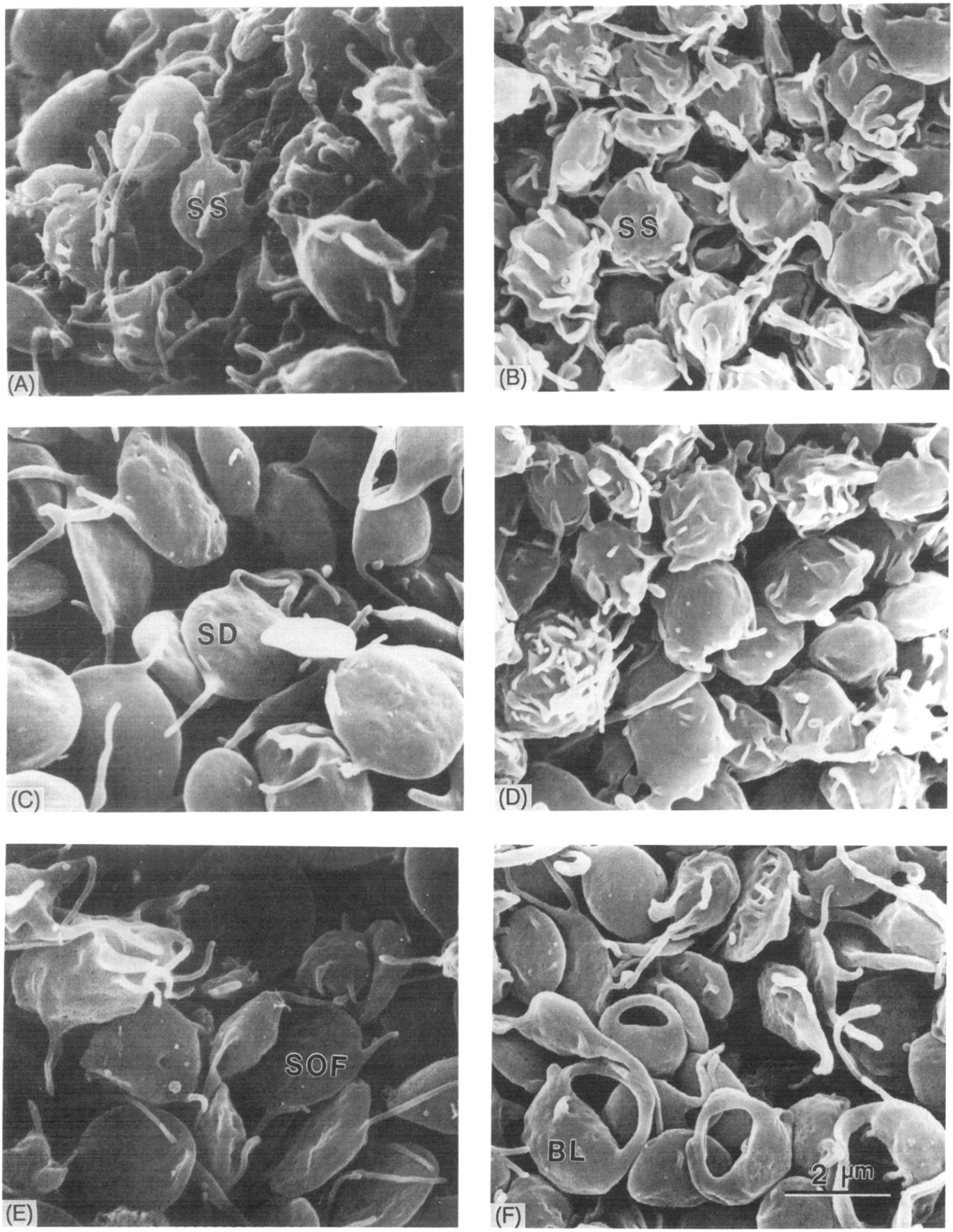


Fig. 7. Scanning electron micrographs showing the time dependent-morphological change of gel-filtered platelets in both cholate (A, C, E) and glycocholate (B, D, F) (×10000). The prewarmed platelets were added with the platelet-saturation concentration (PSC) of either cholate or glycocholate for 1 min (A and B); 5 min (C and D) and 20 min (E and F). The abbreviations in figure are: SS, spiculate sphere cell; SD, spiculate discoid cell; SOF, stretched out flat cell, and BL, basket-like cell. Details of the experiments were given in Materials and Methods.

TABLE II

Effects of various bile salts on platelet function

Data (mean ± S.D.) were taken from at least six experiments in which platelets were preincubated in each detergent for 10 min before addition of agonist. They are expressed as % of control, while 100% of control equals the cell aggregation of gel-filtered platelets stimulated by each kind of agonist. Details of experiments are given in Materials and Methods. Each indicated detergent concentration was its PSC (platelet-saturation concentration).

Agonists	Trihydroxy bile salts			Dihydroxy bile salts	
	cholate (4.8 mM)	glyco-cholate (5 mM)	tauro-cholate (5 mM)	deoxy-cholate (0.97 mM)	chenodeoxy-cholate (0.8 mM)
Collagen	7.5 ± 5.0	1.0 ± 1.0	1.0 ± 1.0	2.0 ± 2.0	2.5 ± 2.0
ADP- fibrinogen	25 ± 2	29 ± 2	23 ± 2	48 ± 3	45 ± 2
Phorbol ester	78 ± 3	72 ± 3	75 ± 3	92 ± 4	95 ± 3
Thrombin	8.0 ± 2.0	1.0 ± 1.0	1.0 ± 1.0	98 ± 5	80 ± 5

might cause membrane vesiculation before cell lysis, since the surviving platelets at the lytic concentration showed filapodia with enlarged tips (Fig. 5D).

In glycocholate, platelets changed morphology to four cell types, i.e., spiculated sphere; stretched-out spiculated disc; basket-like shape and small sphere (1.5 μm in diameter) (Fig. 6). The distribution of these four cell types was also both dose- and time-course-dependent (Figs. 7B, 7D and 7F). A 10 min incubation of platelets at various concentrations resulted in the cells becoming stretched-out spiculate discs at 1 mM (Fig. 6A); spiculated basket-like shapes at 5 mM (Fig. 6C); and small spheres at 15 mM (Fig. 6D). The surviving cells at 45 mM were also small spheres (Figs. 6E). The time-dependent effect on the morphology of platelets at PSC showed that the cells were all spiculate spheres at 1 min (Fig. 7B) and became either stretched-out spiculate discs or spiculated basket-like shapes at 10 min (Fig. 6C), and the filapodia disappeared at 20 min (Fig. 7F). Hence, the results indicate that cholate and its conjugated derivatives had different effects on platelet morphology.

Effect on platelet functions by cholate, glycocholate and taurocholate

Platelets at the PSC of these trihydroxy bile salts failed to respond to both thrombin and collagen (Table II). These detergents also inhibited the response to ADP-fibrinogen and to phorbol ester by 75% and 25%, respectively. Since deoxycholate failed to inhibit responses both to thrombin and phorbol ester (Ref. 6 and Table II), another dihydroxyl bile salt chenodeoxycholate (3α,7α-dihydroxy-5β-cholan-24-oic acid) was used and also showed no inhibition of responses to thrombin and phorbol ester. The physical properties of chenodeoxycholate in solubilizing platelets were similar to deoxycholate (Table I). When platelets were washed after treatment with these trihydroxy bile salts, the washed cells fully recovered the response to thrombin (data not shown). When 5 u/ml thrombin were incubated in 250 mM of each of these trihydroxy bile salts at room temperature for 1 h and then dialyzed against Hepes Tyrode's buffer at 4°C overnight, the pretreated thrombin exhibited the similar stimulation of platelet aggregation as freshly prepared thrombin (data not shown). It indicates that these bile salts did not denature thrombin. Hence, the inhibition of thrombin response by the primary bile salts appeared to require all three hydroxyl groups on the cholanoic ring of the molecule.

Discussion

The study demonstrates that the trihydroxy bile salts cholate and its conjugated derivatives exhibit differential solubilizations of human platelets (Fig. 1 and Table I). Cholate ruptures the membrane in an all-or-none process, which is evident from the comparable percentages among the concentration-dependent curves of: (1) components solubilization; (2) marker leakiness; and (3) the change in optical absorbance of platelet suspension (Fig. 1A). In addition, the composition of phospholipid species in the solubilized fraction is similar to that in whole platelets (data not shown). The cholate-solubilization of platelets is comparable to those by the dihydroxy bile salts (Ref. 6 and Table I). On the contrary, the conjugated derivatives appear to cause shedding of both proteins and lipids prior to causing leaking of lactate dehydrogenase (Figs. 1B and 1C). The shed lipids form vesicles which can be separated from the shed proteins by centrifugation (Fig. 2). The vesicles have a similar phospholipid composition as whole cell (data not shown). It is different from the shedding of erythrocyte membranes by these conjugated salts, in which the shed lipids only derived from the outer leaflet of erythrocyte membrane [30]. These cholate derivatives gradually chop off cell membrane

pieces reducing the size of the cell (Figs. 6D and 6E). Hence, the derivatives cause the increase in the light scattering activity of cell suspension (Figs. 1B and 1C).

The PSC's of these trihydroxy bile salts are similar, while the MCPLC's of the conjugated derivatives are 5-fold larger than that of cholate (Table I), meaning the milder detergent action of these derivatives as suggested in the previous report [31]. The ratio of PSC/CMC among these trihyroxy and dihydroxy bile salts are comparable. These results are also comparable to those found in liposomes [32]; human erythrocytes [30,33,34]; lymphocytes [6]; and hepatic microsomes [4]. Hence, the mode of insertion of both trihydroxy and dihydroxy bile salts may be in dimeric form [3,7].

In the prelytic stage, platelets in these bile salts show no increase in cytosolic free calcium concentration (data not shown). Hence, the morphological changes of platelets in these bile salts may be a direct bile salt effect (Figs. 4–6). The bilayer couple hypothesis has been used to explain the effect of membrane-active agents on platelet membrane morphology [7]. The spiculated platelet form is due to an accumulation of solute in the membrane outer leaflet, while the spherical platelet form is due to the solute concentrating in the inner leaflet. Platelets in these bile salts all appear to change from the initial disc to the final shape as a stretched-out flat form with pseudopods plus filapodia. Hence, these three bile salts may concentrate more in the outer leaflet than in the inner one. This phenomenon was also found in erythrocytes [30,33,34]. The morphological studies also show that these bile salts may affect the organization of cytoskeleton molecules, since platelets in these bile salts deformed to several different shapes (Figs. 5–7, and Ref. 35).

The trihydroxy bile salts inhibit platelet functions more strongly than the dihydroxy bile salts (Table II). Specially the former completely inhibits the thrombin response while the latter does not. The inhibitory effect of these trihydroxy bile salts on thrombin reponse is not due to the membrane shedding, since: (1) cholate does not cause shedding; and (2) washing the platelets after treatment with the conjugated derivatives resulted in the recovery of platelet responses to thrombin (data not shown). An explanation might be that the membrane perturbation by trihydroxy bile salts may be different from that by dihydroxy bile salts. In the blood of patients suffering obstractive jundice, the levels of both cholate and chenodeoxycholate are about 0.1 mM [36]. These patients may have the risk of abnormal hemostasis.

In summary, the study shows the difference in the solubilization of platelet between cholate and its conjugated derivatives. These trihydroxy bile salts inhibit platelet response to thrombin which does not occur in the dihydroxy bile salts.

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