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Research paper

Enhancing effect of 5 α-cyprinol sulfate on mucosal membrane permeability to sodium ampicillin in rats

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Abstract

Effect of 5 α-cyprinol sulfate, a bile alcohol sulfate specific to carp bile, on rectal membrane permeability to sodium ampicillin (AMP Na) was examined in rats. AMP Na is not easily absorbed through rat rectal membrane without aid. 5 α-Cyprinol sulfate significantly enhanced the rectal membrane permeability to AMP Na even at a low concentration (6.25 mM), though sodium taurocholate needed a higher concentration (25 mM). Co-administration of phosphatidylcholine significantly suppressed the enhancing action of both sodium taurocholate and 5 α-cyprinol sulfate. On the other hand, calcium ion did not suppress the action of 5 α-cyprinol sulfate, although it did clearly suppress the action of sodium taurocholate. In conclusion, 5 α-cyprinol sulfate was found to have a potent enhancing effect on mucosal membrane permeability to water-soluble compounds. The enhancing mechanism of 5 α-cyprinol sulfate appeared to be different from that of sodium taurocholate.

Keywords: Bile alcohol; 5 α-Cyprinol sulfate; Membrane permeability; Sodium ampicillin; Physicochemical property

1. Introduction

The bile salts naturally observed in the bile of mammals are mostly mono-, di- or trihydroxy derivatives of cholanoic acid conjugated with taurine or glycine. These endogenous C_{24} acids are made biosynthetically from a C_{27} sterol, cholesterol, in a process involving loss of the terminal three carbon atoms in the side chain. In contrast, bile salts of some evolutionarily primitive animals such as carp and frogs are mostly neutral alcohol' conjugated with sulfuric acid and/or higher bile acids ($>C_{24}$) in place of the usual C_{24} bile acids [1]. These bile alcohol' and higher bile acids are likely intermediates in the biosynthesis of the C_{24} bile acids from cholesterol. A variety of bile alcohol' and higher bile acids, however, have also been detected to some extent, even in healthy humans, mainly as glucuronide conjugates [2–4]. Also, greater amounts of such bile salts are excreted by patients with cerebrotendinous xanthomatosis, acute hepatitis, chronic hepatitis or cirrhosis [5–9].

In the intestinal lumen of mammals, bile salts by stimulating the action of intestinal lipases and/or by forming mixed micelles have many important roles as physiological surfactants in the digestion and absorption of fats including lipid-soluble vitamins and exogenously administered drugs [10,11]. Also, bile acids including their conjugated forms act on the biomembrane directly causing mucosal membrane permeability to many drugs, including water-soluble compounds, and sometimes damage gastrointestinal mucosal barrier [12–16]. Compared with bile acids, little is known about bile alcohol. Free bile alcohol, bile alcohol 3-sulfate, and bile alcohol 3-glucuronide given intraduodenally are absorbed by the intestine in rats, but their extent of intestinal absorption are significantly lower than that of taurocholate [17]. A bile alcohol possessing a sulfuric acid ester group on the side chain is actively transported through the everted gut sacs of rat ileum, as are bile acid conjugates. In contrast, there are many reports available regarding the toxic effects of the ingesting animal bile juices, especially carp bile juice [18–22]. Although it is believed in some cultures that eating the gallbladder of the grass carp improves visual acuity, ingestion of raw carp bile induces toxic acute renal failure and hepatitis in humans. In severe cases, ingestion of raw carp bile kills experimental animals causing a decrease in blood pressure and an increase in plasma potassium, hydrogen ions, blood urea nitrogen and hematocrit. The toxin in carp bile is now identified as 5 α-

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cyprinol sulfate (5 α-cholestane-3α, 7α, 12α, 26, 27-pentol 26 (or 27)-sulfate), an alcohol specific to carp bile [18].

In the present study, we examined the effect of 5 α-cyprinol sulfate on the mucosal membrane permeability to sodium ampicillin (AMP Na) in rats. AMP Na was used as a model compound which is not easily absorbed through rectal membrane in animals and humans without aid, because of its low lipophilicity [12,23]. For comparison, sodium cholate, sodium taurocholate, cholic acid 3-sulfate, and sodium chenodeoxycholate were also employed.

2. Materials and methods

2.1. Materials

AMP Na (912 μg/mg) was obtained from commercial sources and used without further purification. Cholic acid and chenodeoxycholic acid were purchased from Sigma Chemical Company (St. Louis, MO). These bile acids were transformed to sodium salts by reaction with an equimolar amount of sodium hydroxide in a small volume of deionized water. The sodium taurocholate was purchased from Nakarai Tesque (Kyoto, Japan). 5 α-Cyprinol sulfate was extracted from carp bile in this laboratory in the same manner as reported previously [24,25]. Cholic acid 3-sulfate was synthesized [17,26]. All bile salts used were confirmed to be chromatographically pure. Other reagents such as cholesterol and L-α-phosphatidylcholine dipalmitoyl used were of the highest grade available.

2.2. Physicochemical properties

2.2.1. Lipophilicity determination

The R_m value was selected as a measure of lipophilicity. The R_m value of bile salts was measured in a similar manner as reported, with a small modification [12]. Briefly, aliquots (10 μl) of methanol solutions of bile salts were spotted on KC_{18} reversed phase TLC plates (Whatman), and the plates were developed with a mixture of pH 7.4, 1/15 M phosphate buffer solution and methanol. The R_m value was calculated from the following equation: $R_m = \log (1/R_f - 1)$, where the R_f value was obtained with each developing mixture. A plot of the R_m values against the concentrations of methanol in the developing mixture was linear in the range 55–80% methanol, except in the case of cholic acid 3-sulfate. The theoretical R_m values corresponding to 0% methanol were obtained by extrapolation.

2.2.2. Hemolytic activity

The hemolytic activity of bile salts was measured employing sheep blood in the same manner as reported previously [12]. Briefly, erythrocytes of sheep blood were suspended in pH 7.4 isotonic phosphate buffered saline at a concentration of 10 v/v%. To 0.5 ml of erythrocytes suspension, 0.5 ml of isotonic phosphate buffered saline containing a bile salt at various concentrations in the range 1–140 mM, was added. The mixture was incubated for 30 min at 37°C and then centrifuged for 20 min at 3000 rev./min. The supernatant was diluted appropriately with 0.9% NaCl solution, and the concentration of hemoglobin in the supernatant was determined spectrophotometrically at 410 nm. A 100% hemolyzed sample was prepared by adding 4.5 ml of purified water to each 0.5 ml of erythrocytes suspension, and the hemolyzed solution was diluted 40 times with pH 7.4 isotonic phosphate buffered saline.

2.2.3. Solubilizing activity

Solubilizing activity of bile salts in vitro was measured by determining the solubility of cholesterol in pH 7.4, 1/15 M phosphate buffer solution containing a bile salt at a concentration of 12.5 mM. An excess amount (approximately 100 mg) of cholesterol was added to 2 ml of buffer solution containing a bile salt, and the suspension was sonicated for 1 min and shaken mechanically overnight in a room kept at 25°C. After solubility equilibrium was established, an aliquot was filtered through 0.45 μm filters (HA type, Millipore). The concentration of cholesterol in the filtrate was determined by the use of a commercially available analytical kit of cholesterol (Cholesterol C Test-Wako, Wako Pure Chemical Ind., Ltd., Osaka, Japan).

2.3. Effect of bile salts on membrane permeability in vivo

Male Wistar rats weighing 220–300 g were fasted overnight with free access to water prior to the experiments. The effect of bile salts on mucosal membrane permeability was evaluated by determining the rectal absorption of AMP Na in the presence of a bile salt in the same manner as reported previously [12]. Experiments with animals were performed in accordance with the Guide for Animal Experimentation, Hiroshima University and the Committee of Research Facilities for Laboratory Animal Sciences, Faculty of Medicine, Hiroshima University.

2.3.1. Rectal administration

Each fasted rat was anesthetized with sodium pentobarbital (30 mg/kg) by intraperitoneal injection and fixed on a water bed kept at 37°C to maintain normal body temperature during experiments. The rectum was exposed by a midline abdominal incision and was cannulated with polyethylene tubing (PE 50, Clay Adams) at the distal end of the colon. The anus was closed with surgical adhesive cement. The length of rectal loop was about 3 cm. Drug solution was prepared by dissolving AMP Na at a concentration of 15 mg/ml and a bile salt at a concentration of 6.25, 12.5, 25, 37.5 or 50 mM in 1/20 M Tris–HCl buffered saline (pH 8.0). The drug solution was introduced into the rectal loop via polyethylene tubing at the volume of 1 ml/kg (15 mg AMP Na/kg). Blood samples were collected from the jugular vein periodically after rectal administration of AMP Na.

Similar experiments were carried out to examine the effect of calcium ion or phosphatidylcholine dipalmitoyl

on the enhancing action of sodium taurocholate and 5 α-cyprinol sulfate. Calcium chloride or phosphatidylcholine dipalmitoyl was dissolved to give a final concentration of 25 or 12.5 mM, respectively, in a pH 8.0 Tris–HCl buffer solution containing AMP Na (15 mg/ml) and a bile salt (25 mM). In in vivo studies, after mixing all ingredients, the osmolarity of the drug solution was adjusted to 280 mOsm/kgH_2O with NaCl, which was determined with Osmotron-10 (Orion-Riken Co., Ltd.).

2.3.2. Release of proteins from rectal membrane in vitro

Each fasted rat was exsanguinated with an extra amount of sodium pentobarbital by intraperitoneal injection, and the rectum was excised to prepare a 3 cm-long everted rectal sac. Each bile salt was dissolved in 1/20 M isotonic Tris–HCl buffered saline (pH 8.0) at a concentration of 25 mM. The bile salt solution (0.4 ml) was introduced inside the everted sac (serosal side), and both ends of the rectal sac were ligated tightly. The sac was then immersed in 4 ml of the same buffer solution containing a bile salt pre-warmed at 37°C. Under bubbling of mucosal medium with CO_2—O_2 mixture gas, the release of proteins into the incubation media was measured periodically for 30 min. The concentration of proteins was determined by the method of Lowry with bovine serum albumin as the standard.

2.4. Analysis

Whole blood taken periodically was diluted six times with deionized water, and the concentration of AMP Na was microbiologically determined by the paper disc method with *Bacillus subtilis* ATCC 6633 as the test organisms. Assay limit of AMP Na in whole blood was 0.075 μg/ml.

Differences among group mean values were assessed by Student' *t*-test. A difference of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Physicochemical properties of bile alcohol

Some physicochemical properties of 5 α-cyprinol sulfate are summarized in Table 1 together with the data of three other bile salts. 5 α-Cyprinol sulfate showed a relatively higher lipophilicity and a greater solubilizing activity for cholesterol compared to those of sodium cholate and sodium taurocholate. These properties of 5 α-cyprinol sulfate would account for its greater hemolyzing activity. Cholic acid 3-sulfate, which has two functional groups in the side chain and the steroid nucleus, showed low lipophilicity and solubilizing activity. The percentage of hemolysis caused by cholic acid 3-sulfate at a concentration of 50 mM was less than 5%.

Table 1
Physicochemical properties of bile salts used in the present study[a]

Bile salts	Lipophilicity (R_m)	ED_{50} Hemolysis (mM)	Solubilizing activity (mg/l)
Sodium cholate	1.30	35.7	46.3
Sodium taurocholate	1.12	55.3	36.7
Cholic acid 3-sulfate	<0.1	N.D.	12.8
5 α-Cyprinol sulfate	1.83	4.3	142.3

[a] Hemolytic activity was determined using 10% sheep erythrocyte suspensions. The solubilizing activity for cholesterol was measured at a concentration of 12.5 mM. N.D. not detected.

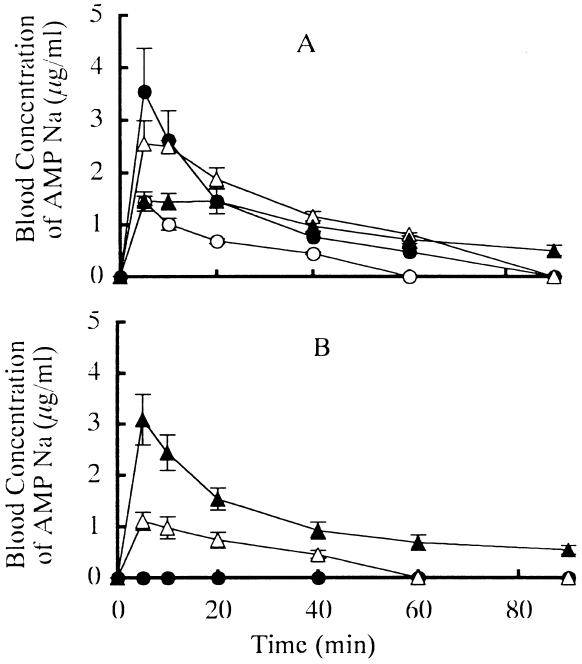

Fig. 1. Enhanced rectal absorption of AMP Na by the presence of 5 α-cyprinol sulfate (A) or sodium taurocholate (B) in rats. The dose of AMP Na was 15 mg/kg. The concentration of a bile salt in the dosing solution was 6.25 (○), 12.5 (●), 25 (△), and 50 mM (▲). The solution containing AMP Na and a bile salt was administered at a volume of 1 ml/kg. Each value represents the mean ± SE of four experiments.

3.2. Effect of 5 α-cyprinol sulfate on rectal membrane permeability to AMP Na

When AMP Na alone was administered rectally at a dose of 15 mg/kg, the blood concentration of AMP Na was less than the detection limit, indicating AMP Na is a compound poorly absorbed through the rectal membrane. Some typical blood concentration-profiles of AMP Na in the presence of a bile salt in the dosing solution are shown in Fig. 1. 5 α-Cyprinol sulfate (Fig. 1A) enhanced the rectal absorption of AMP Na, as did sodium taurocholate (Fig. 1B). Enhanced peak blood concentration and the area under the concentration–time curve from 0 to infinite (AUC) of AMP Na by bile salts are plotted against the concentration of a bile salt in the dosing solution (Fig. 2). Sodium chenodeoxycholate showed the enhancing action even at a lower concentration (6.25 mM). In contrast, the enhancing action of sodium

cholate and sodium taurocholate was detected at a concentration of more than 25 mM. 5 α-cyprinol sulfate, as did sodium chenodeoxycholate, showed an enhancing action at 6.25 mM. Accordingly, their enhancing potencies, evaluated from the onset concentration of enhancing action and enhancing potency at a finite concentration, would be in the order: sodium taurocholate $<$ sodium cholate $<$ 5 α-cyprinol sulfate $<$ sodium chenodeoxycholate. Cholic acid 3-sulfate did not affect the rectal membrane permeability to AMP Na even at a concentration of 50 mM.

3.3. Effect of calcium ion and phosphatidylcholine on 5 α-cyprinol sulfate action

As shown in Figs. 1A and 2, the value of AUC of AMP Na in the presence of 5 α-cyprinol sulfate, as well as the presence of sodium chenodeoxycholate, was likely to remain constant even at a higher concentration of more than 12.5 mM, and the peak blood level of AMP Na was decreased with increase in the concentrations of 5 α-cyprinol sulfate. These phenomena suggest that the absorption rate of AMP Na in the presence of high concentration of 5 α-cyprinol sulfate may be lowered and sustained. To clarify the reason, the solubilizing effect of bile salts on membrane proteins and the inhibitory effect of calcium ion and/or

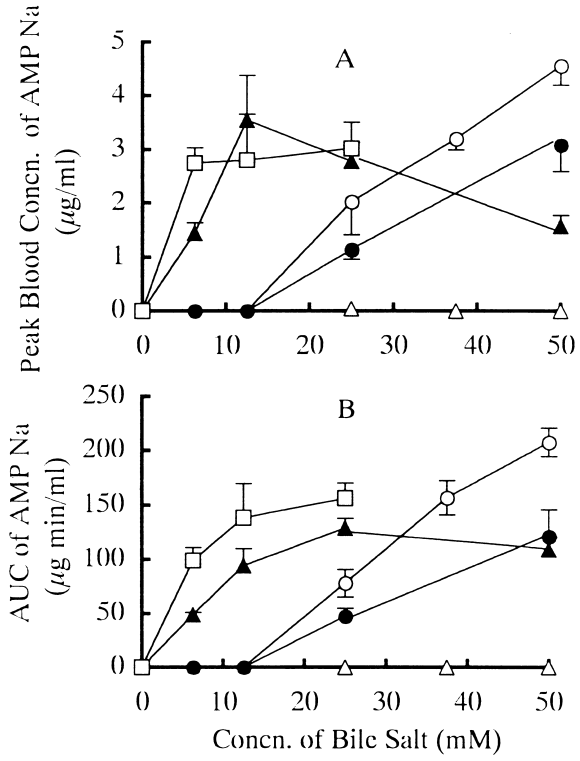

Fig. 2. Plotting of peak blood concentration (A) and AUC (B) of AMP Na in blood against the concentration of a bile salt after rectal administration in rats. The dose of AMP Na was 15 mg/kg. Bile salts used were sodium cholate (○), sodium taurocholate (●), sodium chenodeoxycholate (□), 5 α-cyprinol sulfate (▲), and cholic acid 3-sulfate (△). The solution containing AMP Na and a bile salt was administered at a volume of 1 ml/kg. Each value represents the mean ± SE of four experiments.

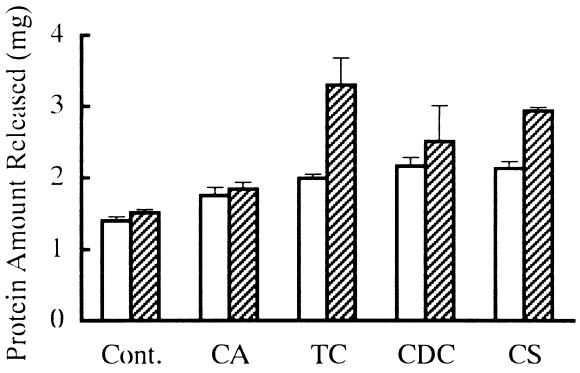

Fig. 3. Release of proteins after 15 (open) and 30 min (slashed bar) from everted rat rectal sac in the absence (cont.) or presence of a bile salt in vitro. CA, sodium cholate; TC, sodium taurocholate; CDC, sodium chenodeoxycholate; CS, 5 α-cyprinol sulfate. A 3 cm-long everted rectal sac was incubated in 4 ml of Tris–HCl buffered saline containing a bile salt (25 mM) at 37°C. All values in the presence of a bile salt were significantly higher than the corresponding value in control at a level of $P < 0.05$. Each value represents the mean ± SE of four experiments.

phosphatidylcholine on the enhancing action of 5 α-cyprinol sulfate were examined. All the bile salts examined increased the release of proteins from the rectal membranes. However, no significant difference in the released amounts of proteins was observed among different bile salts (Fig. 3). In contrast, a significant difference was observed in the effects of calcium ion and phosphatidylcholine (Fig. 4). The enhancing potency of sodium taurocholate was significantly suppressed by the co-administration of either calcium ion or phosphatidylcholine (Fig. 4B). In contrast, the addition of the calcium ion did not suppress, but rather increased the enhancing potency of 5 α-cyprinol sulfate further, although phosphatidylcholine suppressed it significantly (Fig. 4A). These results suggest that there may be some difference in the enhancing mechanisms of sodium taurocholate and 5 α-cyprinol sulfate.

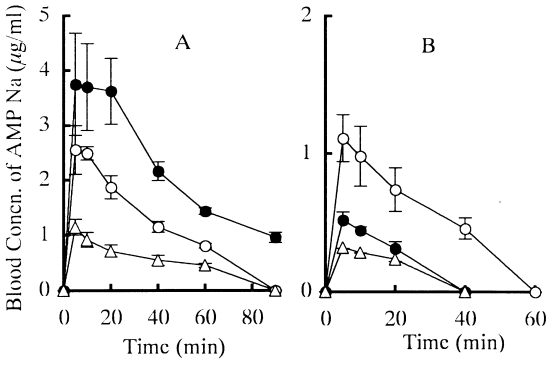

Fig. 4. Effect of coadministration of 25 mM calcium ion (●) or 12.5 mM phosphatidylcholine dipalmitoyl (△) on rectal absorption of AMP Na enhanced by the presence of 25 mM 5 α-cyprinol sulfate (A) or 25 mM sodium taurocholate (B) in rats. (○): A bile salt alone. The dose of AMP Na was 15 mg/ml per kg. Each value represents the mean ± SE of four experiments.

4. Discussion

In the present study, the effect of 5 α-cyprinol sulfate, an alcohol specific to carp bile [18], on the rectal membrane permeability was examined in rats employing AMP Na as a model permeant not easily absorbed. As described already in the Introduction, it has been reported that the ingestion of animal bile juices, especially carp bile juice, causes some severe toxic effects in humans. Also, it is well known that some bile acids including their conjugated forms alter the mucosal membrane permeability to many compounds, including water-soluble compounds, and cause mucosal damage at high concentrations. However, information regarding the action of bile alcohol sulfates on biomembranes has, until now, been lacking. AMP Na is a highly water-soluble compound. The direct interaction between AMP Na and bile salts such as the micelle formation could thus be negligible. Accordingly, the effect of bile salts on the rectal absorption of AMP Na observed in the present study would be derived from the direct action of bile salts on biomembranes.

Previously, we have reported, employing nine bile salts but no bile alcohol', that the bile salts having higher hemolytic activity, higher lipophilicity, and higher values of lipophilicity × calcium ion sequestration capacity such as dihydroxycholates showed higher enhancing effects on rectal absorption of AMP Na in rats [12]. Also, the enhancing potency of dihydroxycholates was significantly suppressed by co-administering calcium or magnesium ions. The affinity of bile salts for calcium ions is involved in their physiological actions, for example, slowing gastric emptying, anti-proteinase activity, activation of lipase, and so on [11,23,27]. Thus, in the present study, the enhancing action of a bile alcohol sulfate, 5 α-cyprinol sulfate, was examined in relation to the physicochemical properties such as lipophilicity, hemolytic activity, and the effect of calcium ions, by comparing them mainly with sodium taurocholate. Sodium taurocholate also possesses a sulfuric acid ester group in the side chain, as well as 5 α-cyprinol sulfate.

The rank order of enhancing potency of bile salts examined was sodium taurocholate < sodium cholate < 5 α-cyprinol sulfate < sodium chenodeoxycholate. Cholic acid 3-sulfate had no such effect (Fig. 2). The greater potency of sodium chenodeoxycholate, a dihydroxy-bile acid, among these five bile salts would be due to its great hemolytic activity, lipophilicity, and calcium ion sequestration capacity, as described above. The greater enhancing effect of 5 α-cyprinol sulfate over of sodium taurocholate would be due to the higher lipophilicity and higher hemolytic activity. In general, the enhancing effect of bile salts can not be accounted for by their solubilizing properties alone. For example, trihydoxycholate conjugated with taurine or glycine have much higher solubilizing activity for lipophilic compounds than those of dihydroxycholic acids, however, their enhancing effects on mucosal membrane permeability are far lower than those of dihydroxy-bile acids [12]. Thus, the lipophilicity of bile salts, or the membrane permeability of bile salts themselves, in addition to the solubilizing activity, would be essential in the modification of membrane permeability. Accordingly, the lesser effect of cholic acid 3-sulfate would be accounted for by the lesser lipophilicity and lesser solubilizing activity.

The enhancing potency of sodium cholate and sodium taurocholate was increased with increase in concentration, at least in the concentration range from 12.5 to 50 mM. On the other hand, upon addition of 5 α-cyprinol sulfate or sodium chenodeoxycholate at concentrations of more than 12.5 mM, their enhancing efficacy remained almost constant as evaluated by the AUC values of AMP Na. The maximal rectal bioavailability of AMP Na enhanced by these bile salts was approximately 30%. These phenomena indicate that there is a saturation in their enhancing potencies. To examine the reason, the production of bile salts for the release of membrane proteins from the rat rectum, which would be related to the morphological damages of the membrane, was compared in vitro. All bile salts examined increased the release of proteins, but only by a small degree. Also, there was no difference among them in the amounts of proteins released. Thus, the release of proteins from the rectal membrane in the presence of bile salts, and/or the mucosal damage caused by these bile salts, would be ruled out of the main mechanism of their enhancing action.

As to the action of the bile salts in altering the permeability of mucosal membrane, interaction of bile salt micelles with membrane lipid, particularly phospholipids, has been suggested, in addition to many other effects such as producing release of deoxyribonucleic acid, cholesterol, and increasing the forward diffusion of sodium ions [28–31]. Also, it has been reported that bile acids including their conjugated forms, as well as disodium ethylene diamine tetraacetate (EDTA), decrease the electrical resistance of rat jejunal membrane and increase the mucosal-to serosal flux of sulfanil acid, a water-soluble compound, probably via a paracellular route [16]. Many other adjuvants that modify the membrane permeability to water-soluble compounds such as *N*-acylamino acids, saponines and medium chain fatty acids have a calcium-chelating ability, as does EDTA, and their enhancing potencies are remarkably suppressed by the presence of calcium ion [32–34]. Previously, we proposed that two different paracellular mechanisms, one being calmodulin-dependent and the other, protein kinase C (or calcium chelation)-dependent, can explain the permeation-enhancing effect of decanoic acid across Caco-2 cell monolayers [35]. The effect of phosphatidylcholine and calcium ion on the enhancing effect of sodium taurocholate and 5 α-cyprinol sulfate was also examined based on these considerations (Fig. 4). The presence of phosphatidylcholine in the dosing solution suppressed the enhancing potency of both 5 α-cyprinol sulfate and sodium taurocholate. In contrast, the addition of calcium ion did not suppress, but rather increased the enhancing potency of 5 α-cyprinol sulfate further, although

it did suppress the potency of sodium taurocholate. Taken together, the saturable enhancing potency of 5 α-cyprinol sulfate may be accounted for as follows: 5 α-cyprinol sulfate extracts phospholipids from the membrane and the activity of 5 α-cyprinol sulfate is decreased by the formation of mixed micelles. The protein extracting activity of 5 α-cyprinol sulfate may increase at a higher concentration, and thereby result in saturation of the enhancing action. The mechanism of the possible interaction of 5 α-cyprinol sulfate with calcium ions is not clear at present. Further investigation is necessary to understand the mechanism of 5 α-cyprinol sulfate.

In conclusion, 5 α-cyprinol sulfate present in carp bile was found to have a potent enhancing effect on mucosal membrane permeability to AMP Na, a water-soluble compound, even at a low concentration in rats. It was suggested that there is some difference in the mechanism of action between 5 α-cyprinol sulfate and sodium taurocholate. The action of 5 α-cyprinol sulfate in altering the membrane permeability would likely be related to the toxic effects of ingestion of raw carp bile in humans.

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