J. Biochem. **123**, 107-114 (1998)

Inhibition of Pancreatic Elastase by Sulfated Lipids in the Intestinal Mucosa[1]

Nobuko Ito,* Yuriko Iwamori,† Kazuo Hanaoka,* and Masao Iwamori†,[2]

*Departments of *Anesthesiology and †Biochemistry, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113*

Received for publication, August 4, 1997

Sulfated lipids, cholesterol sulfate (CS) and I^3SO_3-GalCer, are commonly present in the epithelia of the digestive tracts of pigs, humans, rabbits, and rats. CS was the only sulfated lipid in the esophageal epithelia of these mammals, and I^3SO_3-GalCer, together with CS, was detected in the epithelia of the gastrointestinal tracts, at a concentration higher than 0.05 μmol per gram of dry weight. Although no sulfated lipids were present in the pancreatic duct, they were found in relatively high concentrations in the duodenal, jejunal, and ileal epithelia. To elucidate the functional significance of sulfated lipids in the digestive tract, we determined the effect of CS and I^3SO_3-GalCer on the activities of pancreatic and *Pseudomonas aeruginosa* elastases and found that both characteristically inhibited the pancreatic elastase but not the *P. aeruginosa* elastase. Desulfation of CS and I^3SO_3-GalCer abolished their inhibitory activity, and other membrane constituents including free fatty acids, phospholipids, and gangliosides failed to inhibit pancreatic elastase. In addition, steroid sulfates, such as dehydroepiandrosterone sulfate and pregnenolone sulfate, did not exhibit any inhibitory activity toward pancreatic elastase, indicating that the sulfate group and a suitable hydrophobic side chain are required in the inhibition of elastase. Inhibition of elastase by sulfated lipids occurred in a dose-dependent manner, and the molar ratios of CS and I^3SO_3-GalCer to elastase at which the enzyme activity was inhibited to 50% of the maximum level were 6:1 and 9:1, respectively. CS-treated elastase had the same K_m and a lower V_{max} compared with the untreated enzyme, and sulfated lipids were observed to bind tightly to the enzyme, suggesting irreversible inhibition. Thus, CS and I^3SO_3-GalCer in the digestive tracts of mammals were shown to function as epithelial inhibitors of pancreatic elastase.

Key words: cholesterol sulfate, gangliosides, pancreatic elastase, *Pseudomonas aeruginosa* elastase, sulfatide.

Pancreatic elastase [EC 3.4.21.36] is a serine protease in the pancreas of mammals which, together with trypsin and chymotrypsin, is involved in the digestion of dietary proteins. Its zymogen, proelastase, is secreted from pancreatic acinar tissues (*1*) and is converted to active elastase by tryptic cleavage in the duodenum (*2*). The active elastase is capable of digesting elastin, the elastic fibrous protein of connective tissues, as well as several proteins on the C-terminal side of amino acids bearing uncharged nonaromatic side chains, and this substrate specificity is different from those of pancreatic trypsin and chymotrypsin (*3*). Thus, the pancreatic proteases digest multiple proteins from endogenous and exogenous sources and are the major class of proteases in the intestine. However, regulation of their activities is also important to prevent autolysis of tissues. Endogenous protein inhibitors have been shown to regulate elastase activity (*4-9*), and heat-treated ovalbumin and elastin-derived peptide have also been shown to inhibit the activity (*10-12*). However, although the possible involvement of these protein inhibitors in the activity of neutrophil elastase has been well characterized in relation to cystic fibrosis and gastric ulcers (*4, 13, 14*), their inhibition of pancreatic elastase in the digestive tract has not yet been clarified. During a study on the functional significance of membrane constituents in the digestive tract, we recently found an inhibitory activity of sulfated lipids toward pancreatic trypsin and chymotrypsin (*15*), indicating that sulfated lipids in the epithelial cells of the digestive tract play a role in the inhibition of the activities of several proteases, probably for protection from tissue injury by proteases. To evaluate this finding, we

[1] This work was supported by a Grant-in-Aid for Scientific Research, No. 08680646, from the Ministry of Education, Science, Sports and Culture of Japan.

[2] To whom correspondence should be addressed. Tel: +81-3-3812-2111 (Ext. 3445), Fax: +81-3-3813-8732, E-mail: iwamori@m.u-tokyo.ac.jp

Abbreviations: BANP, butoxycarboxyl-alanine-4-nitrophenol ester; CS, cholesterol sulfate; Sul, sulfatide, I^3SO_3-GalCer, SO_3-3Galβ1-1′ ceramide; GM3, II^{3}NAα-LacCer, NAα2-3Galβ1-4Glcβ1′ceramide; NA, sialic acid; TLC, thin-layer chromatography; FABMS, fast atom bombardment mass spectrometry; HEPES, *N*-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

determined the effect of various lipids on the activity of pancreatic elastase.

MATERIALS AND METHODS

Tissues—Rabbits (New Zealand White, 2 months old) and rats (SD, 2 months old) were provided by Japanese Biological Materials, Tokyo, and the digestive tracts of pigs (6 months old) were purchased from the local slaughterhouse. Epithelia of human digestive tracts were obtained from the University Hospital, The University of Tokyo.

Materials—CS was synthesized by sulfation of the 3β-hydroxy group of cholesterol (Wako Chemicals, Tokyo) with pyridinium sulfate. I^3SO_3-GalCer, $II^3NA\alpha$-Gg_4Cer, $II^3NA\alpha IV^3NA\alpha$-$Gg_4Cer$, and $II^3NA\alpha_2IV^3NA\alpha$-$Gg_4Cer$, and LacCer and $II^3NA\alpha$-LacCer were prepared from human brain and placenta, respectively, in our laboratory. Phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, and sphingomyelin were gifts from Dr. Soda, Nihon Shoji, Osaka. The following chemicals were purchased from commercial sources: dehydroepiandrosterone, dehydroepiandrosterone sulfate, pregnenolone, pregnenolone sulfate, estrone, estrone sulfate, testosterone, testosterone sulfate, sodium dodecyl sulfate, cholesterol, oleic acid, triolein, and butoxycarbonyl-alanine-4-nitrophenol ester (BANP) from Sigma (St. Louis, MO, USA); elastase (EC 3.4.21.36, porcine pancreas) from Boehringer Mannhein (Germany); elastase (*Pseudomonas aeruginosa*) from Wako Chemicals (Tokyo); orcein-elastin from Nacalai Tesque (Kyoto). ^{35}S-labeled CS was synthesized using cholesterol, phosphoadenosine phospho-^{35}S-sulfate (74.0 GBq/mmol, New England Nuclear, Boston, MS, USA), and the cytosol fraction of rabbit uterine endometrium according to the method described previously (*16*). Its specific activity after purification was 0.5 GBq/mmol.

Determination of Acidic Lipids in the Digestive Tracts of Pig, Human, Rat, and Rabbit—The epithelial layers of pig, human, rat, and rabbit digestive tracts were removed from the tunica muscularis, homogenized in water with a Polytron homogenizer, then lyophilized. Total lipids were extracted from the lyophilized powder with chloroform/methanol/water (20 : 10 : 1, 10 : 20 : 1, and 1 : 1, by volume), and the combined extracts were applied onto a column packed with DEAE-Sephadex A-25 (acetate form; Pharmacia, Uppsala, Sweden). After elution of the unabsorbed lipids with 3 volumes of chloroform/methanol (1 : 1, by volume) and 1 volume of methanol, the acidic lipids absorbed on the column were eluted with 10 volumes of 0.3 M sodium acetate in methanol, then saponified with 0.5 M sodium hydroxide in methanol at 40°C for 30 min to cleave the ester-containing acidic phospholipids (*17*). After removal of salts by dialysis, the solution was evaporated to dryness and the acidic lipids thus obtained were dissolved in chloroform/methanol (1 : 1, by volume). The acidic lipids were chromatographed on a TLC plate (E. Merck, Darmstadt, Germany) with the solvent systems of chloroform/methanol/water (65 : 35 : 8, by volume) and chloroform/methanol/acetone/acetic acid/water (8 : 2 : 4 : 2 : 1, by volume) and were detected with cupric acetate-phosphoric acid for organic compounds, ferric chloride-sulfuric acid for steroids, and orcinol-sulfuric acid for glycolipids. Known amounts of CS, I^3SO_3-GalCer, and $II^3NA\alpha$-LacCer were spotted on the same plate, and the density of the spots was determined at a sample wavelength of 420 nm and a control wavelength of 700 nm with a TLC-densitometer (CS-9000; Shimadzu, Kyoto). The lower limit for detection was 0.05 μg and the standard curves were linear up to 1.5 μg.

Determination of the Activity of Elastases from Porcine Pancreas and Pseudomonas aeruginosa—Pancreatic elastase activity was measured with BANP and orcein-elastin as the substrates. For the reaction with BANP, elastase (1 μg) in 90 μl of 50 mM HEPES-NaOH buffer (pH 7.5) was incubated with several lipids in 10 μl of dimethylsulfoxide (DMSO) at 37°C for 30 min, then 100 μl of BANP (3 mM in DMSO) and 800 μl of the above buffer were added. After incubation at 37°C for 10 min, the optical density at 405 nm was measured. The rate of reaction was determined from a calibration curve made with *p*-nitrophenol (Wako Chemicals). For the reaction with orcein-elastin, 10 μg of pancreatic elastase in 100 μl of 30 mM Tris-HCl Buffer (pH 8.0) was incubated with several lipids in 10 μl DMSO at 37°C for 30 min, then 2 mg of orcein-elastin and 590 μl of the above buffer were added. After incubation with shaking at 40°C for 3 h, the reaction was terminated by addition of 200 μl of 0.7 M phosphate buffer (pH 6.0) and the optical density at 590 nm was measured after centrifugation at 1,500 rpm for 5 min. The activities of elastases from *P. aeruginosa* and porcine pancreas were also measured with casein (Sigma) as the substrate. After treatment of the enzyme with several lipids as described above, 2 mg of casein in 200 μl of 10 mM Tris-HCl buffer (pH 7.4) was added, and the solution was incubated at 40°C for 10 min. The reaction was terminated by the addition of 200 μl of 10% trichloroacetic acid, and the optical density at 280 nm was measured after centrifugation at 15,000 rpm for 10 min. Protein concentration was measured by the protein-dye binding method with bovine serum albumin as the standard (*18*).

Enzyme-Linked Immunosorbent Assay—Porcine pancreatic elastase (5 μg) in 500 μl of 50 mM HEPES-NaOH buffer (pH 7.5) was incubated with 25 μg of cholesterol or 25 μg of CS in 20 μl of DMSO at 37°C for 30 min, then 100 μl of the solution was put into each well of a microtiter plate (ICN Biomedical, Auroma, OH, USA). After leaving the plate at 4°C overnight, it was blocked with 50 μl of 1% polyvinylpyrrolidone and 1% ovalbumin in PBS at 37°C for 1 h, then reacted with rabbit anti-porcine pancreatic elastase antibody (Rockland, Gilbertsville, PA, USA) diluted serially with 3% polyvinylpyrrolidone in PBS at 37°C for 1 h. After washing the plate five times with 0.1% Tween 20 in PBS, it was reacted with horseradish peroxidase-conjugated anti-rabbit IgG+IgM antibody (Cappel, Cochraville, PA) diluted 1:500 with 3% polyvinylpyrrolidone in PBS at 37°C for 30 min, then washed five times with 0.1% Tween 20 in PBS. Peroxidase in the plate was visualized by incubation with 5 mM *o*-phenylenediamine (Wako Chemicals) in 25 mM citrate-phosphate buffer (pH 5.5) at 37°C for 10 min, and the optical density at 415 nm was measured with an ELISA reader (Corona, Tokyo). To exclude the possibility that sulfated lipids might inhibit the binding of elastase to the ELISA plate, 2.5 μg of porcine pancreatic elastase was first coated on the plate, then 2 μg of cholesterol, CS, or I^3SO_3-GalCer in 5 μl of DMSO and 45 μl of 50 mM HEPES-NaOH buffer (pH 7.5) was reacted by incubation at 37°C for 30 min. The reactivity of elastase on

the ELISA plate toward anti-elastase antibody was measured as above.

Native and SDS-PAGE—Porcine pancreatic elastase (5 μg) was incubated with 25 μg of ^{35}S-CS (2×10^4 cpm) and analyzed by native and SDS-PAGE with 12% polyacrylamide gels (*19*). Gels were stained with Coomassie brilliant blue and the distribution of radioactivity on the gels was examined by exposing them to X-ray film (NS54T, Kodak, USA).

RESULTS

Sulfated Lipids in the Digestive Tract of Mammals—As shown in Fig. 1, although II^{3}NAα-LacCer was present in relatively high concentrations in both the tunica muscularis and epithelium of the porcine esophagus, CS was preferentially contained in the epithelial layer. Similarly, I^{3}SO$_3$-GalCer in the gastrointestinal tract was present in the epithelial layer, indicating the specific localization of sulfated lipids, CS, and I^{3}SO$_3$-GalCer, in the epithelial layer of porcine digestive tracts. The identities of I^{3}SO$_3$-GalCer and II^{3}NAα-LacCer were confirmed by comparing their mobilities on a TLC plate with those of standard glycolipids and by their reactions with monoclonal anti-I^{3}SO$_3$-GalCer (TCS-1) (*20*) and anti-II^{3}NAα-LacCer (M2590, Meiji, Tokyo) antibodies. CS was characterized by comparing its mobility on TLC with that of standard CS, by its reaction with ferric chloride-sulfuric acid for steroid, and by analysis with a negative ion fast atom bombardment mass spectrometer (FABMS). CS isolated from the esophageal and jejunal epithelia yielded an intense molecular ion, $[M-H]^-$, at m/z 465 and the fragment ion, $[HSO_4]^-$, at m/z 97 by negative ion FABMS, and only cholesterol was detected in the products after solvolysis (*16*). As shown in Table I, CS was present in relatively high concentrations in the esophageal epithelium, and was also detected in the epithelia of gastrointestinal tract, but not in the pancreas or pancreatic duct. I^{3}SO$_3$-GalCer was present in the epithelia of gastrointestinal tract and the pancreas, but not in the pancreatic duct, and a relatively high concentration was observed in the jejunal and ileal epithelia. The same sulfated lipids were distributed in the epithelia of the digestive tracts of humans, rabbits, and rats (Table II), but their concentrations were different among these mammals. In particular, the concentrations of CS in the rat intestinal epithelia were much higher than those of I^{3}SO$_3$-GalCer. Common observations regarding the lipids in the mammalian digestive tracts were that CS was the only sulfated lipids in the esophageal epithelium, and the duodenal, jejunal, and ileal epithelia contained significantly high concentrations of sulfated lipids, either CS or I^{3}SO$_3$-GalCer.

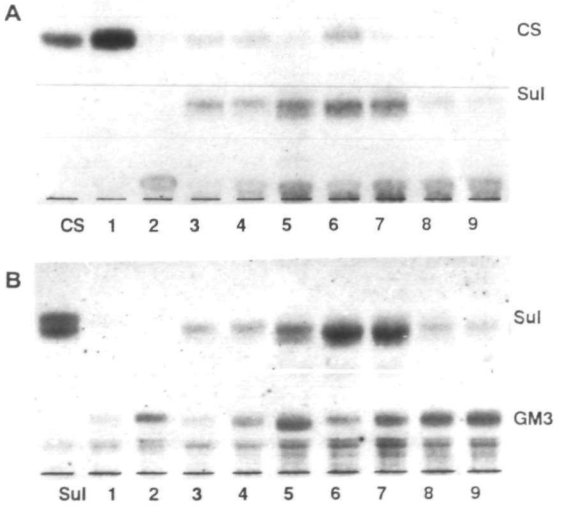

Fig. 1. **TLC of acidic lipids in porcine digestive tract.** For A: Acidic lipids corresponding to 5 mg dry weight were developed on a TLC plate with chloroform/methanol/acetone/acetic acid/water (8 : 2 : 4 : 2 : 1, by volume) and detected with cupric acetate/phosphoric acid reagent. B: Acidic lipids corresponding to 2 mg dry weight were developed with chloroform/methanol/water (65 : 35 : 8, by volume) and detected with orcinol-sulfuric acid reagent. CS, chemically synthesized cholesterol sulfate; acidic lipids from: 1, esophageal epithelium; 2, esophageal tunica muscularis; 3, gastric epithelium (fundus); 4, gastric epithelium (antrum); 5, duodenal epithelium; 6, jejunal epithelium; 7, ileal epithelium; 8, colonic epithelium; 9, rectal epithelium.

Effects of Sulfated Lipids on the Activities of Bacterial and Pancreatic Elastases—We first examined the reaction conditions of pancreatic and *P. aeruginosa* elastases. Pancreatic elastase readily hydrolyzed BANP, casein, and orcein-elastin under the conditions described in the text, while *P. aeruginosa* elastase hydrolyzed casein at a similar rate, but BANP and orcein-elastin at lower rates. Accordingly, we chose BANP, casein, and orcein-elastin as substrates for pancreatic elastase, and casein for *P. aeruginosa*

TABLE I. **Concentrations of acidic lipids in the porcine digestive tracts and the pancreas** (μmol/g of dry tissue weight).

Tissues	Cholesterol	Lipid-bound phosphorus	CS	Sul	GM3
Esophageal epithelium	29.33	29.93	0.62	nd	0.02
Esophageal tunica muscularis	6.28	36.40	nd	nd	0.12
Gastric epithelium (fundus)	49.23	36.14	0.02	0.06	0.09
Gastric epithelium (antrum)	83.62	70.50	0.04	0.10	0.02
Duodenal epithelium	61.08	80.28	tr	0.27	0.19
Jejunal epithelium	58.47	56.16	0.09	0.56	0.09
Ileal epithelium	10.25	52.77	tr	0.63	0.16
Colonic epithelium	38.12	40.60	tr	0.10	0.19
Rectal epithelium	36.38	36.75	tr	0.06	0.20
Pancreas	5.43	–	nd	0.04	0.01
Pancreatic duct (inner layer)	8.27	–	nd	nd	0.04
Pancreatic duct (outer layer)	1.29	–	nd	nd	0.02
Pancreatic juice	0.26	–	nd	nd	nd

nd, not detected; tr, trace amount (less than 0.01 μmol/g of dry tissue weight); –, not determined.

TABLE II. **Concentrations of acidic lipids in the epithelia of the digestive tracts of human, rabbit, and rat** (μmol/g of dry tissue weight).

	Human			Rabbit			Rat		
	CS	Sul	GM3	CS	Sul	GM3	CS	Sul	GM3
Esophagus	1.50	nd	0.02	0.28	nd	0.03	0.72	nd	0.04
Stomach (antrum)	0.24	0.05	0.06	0.05	0.21	0.11	0.07	0.01	0.08
Duodenum	0.60	0.22	0.22	0.64	0.56	0.18	0.64	0.01	0.18
Jejunum	0.32	0.50	0.13	0.89	0.36	0.06	0.95	tr	0.18
Ileum	0.22	0.06	0.12	0.16	0.03	0.08	1.39	tr	0.20
Colon	0.02	0.04	0.19	0.23	0.08	0.16	0.94	tr	0.10

nd, not detected; tr, trace amount (less than 0.01 μmol/g of dry tissue weight).

elastase. The activities of pancreatic and bacterial elastases with the above substrates were not altered on incubation in the buffer containing up to 16% DMSO at 37°C for 1 h. As shown in Fig. 2, CS suspended in 50 mM HEPES-NaOH buffer (pH 7.5) inhibited the activity of pancreatic elastase to the same level as CS in DMSO, and the maximum inhibition was obtained by incubation at 37°C for 30 min;

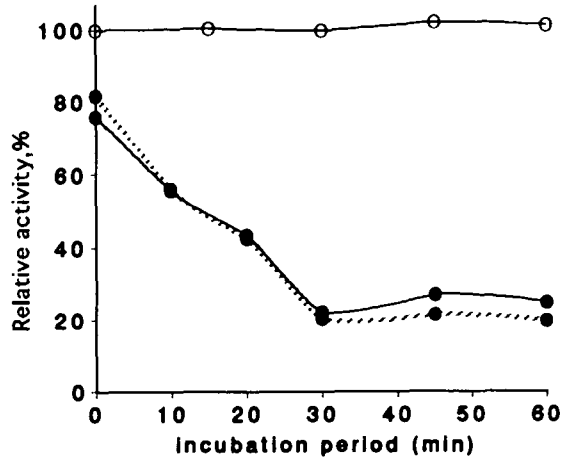

Fig. 2. **Effect of preincubation of pancreatic elastase with CS and cholesterol on its activity.** Porcine pancreatic elastase (1 μg) in 90 μl of 50 mM HEPES-NaOH buffer (pH 7.5) was incubated either with 5 μg of CS in 10 μl DMSO (●–), with 5 μg of CS suspended in the same buffer (●···), or 5 μg of cholesterol (○–) in 10 μl DMSO at 37°C for various times, and the enzyme reaction was initiated by addition of 100 μl of BANP in DMSO and 800 μl of the same buffer. After incubation at 37°C for 10 min, the optical density at 405 nm was measured.

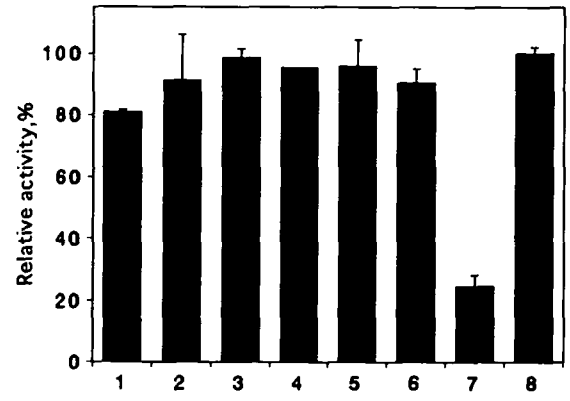

Fig. 3. **Effect of various lipids on the activity of porcine pancreatic elastase.** Porcine pancreatic elastase (1 μg) in 90 μl of 50 mM HEPES buffer (pH 7.5) was incubated with 10 μg of various lipids in 10 μl of DMSO at 37°C for 30 min, and the enzyme reaction was initiated by addition of 100 μl of BANP in DMSO and 800 μl of the same buffer. After incubation at 37°C for 10 min, the optical density at 405 nm was measured. 1, oleic acid; 2, triolein; 3, phosphatidyl choline from egg yolk; 4, distearoyl phosphatidyl ethanolamine; 5, phosphatidyl serine; 6, sphingomyelin; 7, CS; 8, no inhibitor.

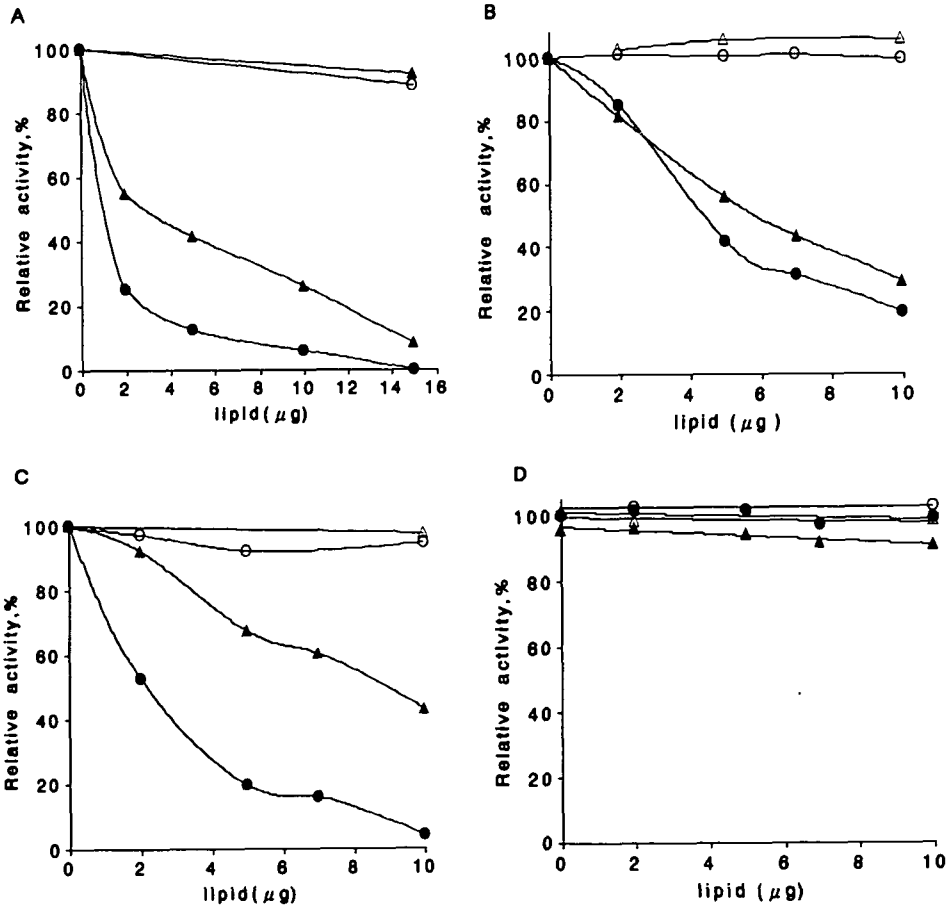

Fig. 4. **Effect of cholesterol (○), GalCer (△), CS (●), and I^3SO_3-GalCer (▲) on the activities of pancreatic (A, B, C) and bacterial elastases (D).** As described in the text, either porcine pancreatic elastase (1 μg) or *P. aeruginosa* elastase (1 μg) in 10 μl of 50 mM HEPES buffer (pH 7.5) was incubated with various amounts of lipids in 10 μl of DMSO at 37°C for 30 min, and the activities were measured with orcein-elastin (A), BANP (B), and casein (C and D) as the substrates.

but the other lipids, particularly hydrophobic lipids such as cholesterol and GalCer, were insoluble in the buffer. Since DMSO could be used to prepare a homogeneous lipid solution without adversely affecting the enzyme activity, we chose DMSO as the solvent for the various lipids and found that cholesterol in DMSO did not inhibit the activity of pancreatic elastase. The effects of several lipids in the mammalian digestive tract on the activity of pancreatic elastase were determined by pretreatment of elastase with the lipids in DMSO at 37°C for 30 min. Of the lipids tested, only CS and I^3SO_3-GalCer inhibited the activity of pancreatic elastase, no inhibition was observed with the following lipids: cholesterol, oleic acid, triolein, phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, sphingomyelin, GalCer, LacCer, $II^3NA\alpha$-LacCer, $II^3NA\alpha$-Gg_4Cer, $II^3NA\alpha IV^3NA\alpha$-$Gg_4Cer$, or $II^3NA\alpha_2IV^3NA\alpha$-$Gg_4Cer$ (Fig. 3). As shown in Fig. 4, pancreatic elastase activities toward orcein-elastin, BANP, and casein were inhibited by sulfated lipids in a dose-dependent manner, but not by desulfated lipids, cholesterol or GalCer. In contrast, the elastase from *P. aeruginosa* was not inhibited by incubation with CS or I^3SO_3-GalCer, or with the desulfated derivatives. Thus, sulfated lipids, as common constituents in the mammalian digestive tract, showed inhibitory activity toward pancreatic elastase but not toward bacterial elastase. Inhibition of pancreatic elastase by CS was accompanied by reduction of the V_{max} without change of the K_m, indicating that CS does not affect the affinity between the enzyme and the substrate (Fig. 5). The concentration at which 50% of the elastase activity toward orcein-elastin was inhibited was 2.14 μM for CS and 3.48 μM for I^3SO_3-GalCer, and the molar ratios of CS and I^3SO_3-GalCer to elastase were 6:1 and 9:1, respectively. The reactivity of elastase with anti-elastase antibody was completely lost following incubation of the enzyme (5 μg) with 25 μg of CS, suggesting a masking or a conformational change of the antigenic determinant of elastase by CS (Fig. 6). In addition, after incubation of elastase (M.W. 25.9 kDa) with ^{35}S-CS, elastase and ^{35}S-CS were retained on the top of a native polyacrylamide gel, but they were separated by SDS-PAGE, on which ^{35}S-CS migrated to the position of the bromphenol blue used as a marker for the gel front, suggesting the formation of a high-molecular-weight aggregate of pancreatic elastase in the presence of CS (Fig. 7). Figure 8 shows the effects of several compounds structurally related to CS on the activity of elastase. Steroid sulfates such as dehydroepiandrosterone sulfate, pregnenolone sulfate, estrone sulfate, and testosterone sulfate, even at 30 μM, did not inhibit porcine pancreatic elastase, and neither did gangliosides and sodium taurocholate at 30 μM, while SDS slightly

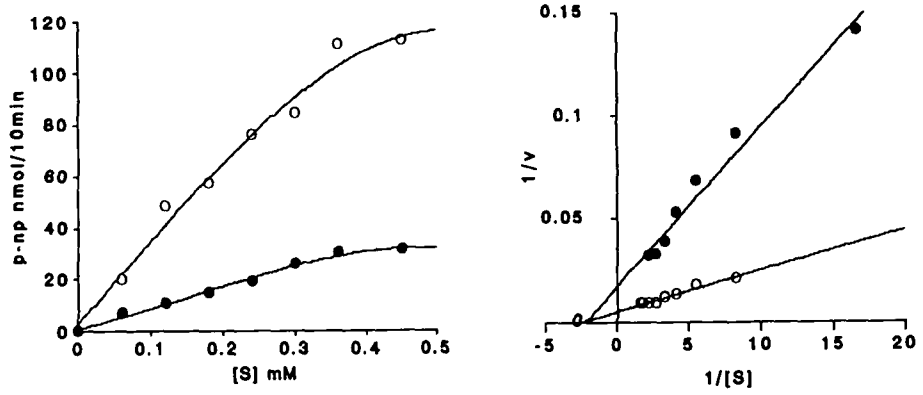

Fig. 5. **Activities of porcine pancreatic elastases preincubated in the presence and absence of CS as a function of substrate concentration.** Porcine pancreatic elastase (1 μg) in 90 μl of 50 mM HEPES buffer (pH 7.5) was incubated with either 10 μl of DMSO (○) or 5 μg of CS in 10 μl of DMSO (●) at 37°C for 30 min, and the activities were determined with various concentrations of BANP according to the method described in the text.

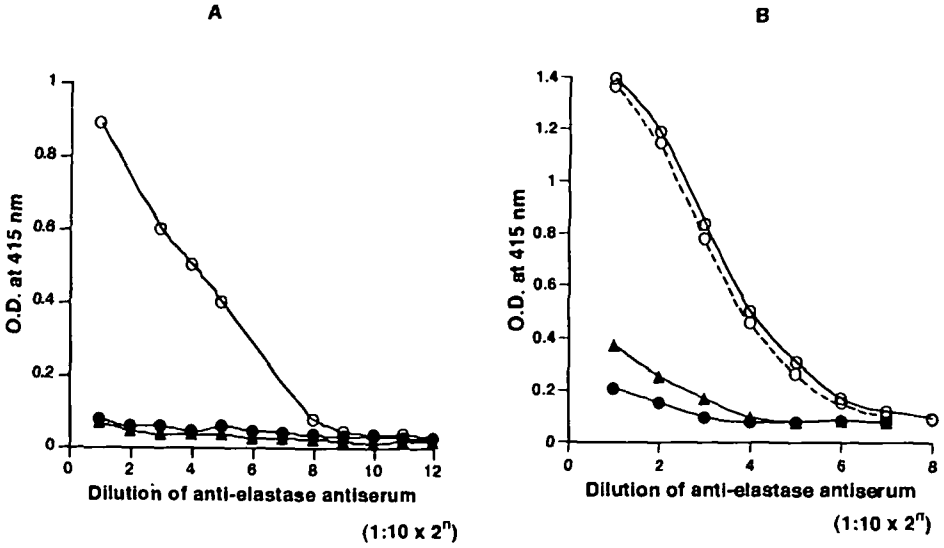

Fig. 6. **ELISA of porcine pancreatic elastase pre- and post-reacted with sulfated lipids.** A: Porcine pancreatic elastase (5 μg) in 500 μl of 50 mM HEPES-NaOH buffer (pH 7.5) was incubated with 25 μg of CS (●) or I^3SO_3-GalCer (▲) in 20 μl of DMSO at 37°C for 30 min and 100 μl of the solution was put into each well of a microtiter plate. ○, DMSO alone. B: A microtiter plate was coated by incubation with 2.5 μg of elastase in 50 μl of HEPES-NaOH buffer at 4°C overnight, then reacted with a mixture of 5 μl of DMSO and 45 μl of HEPES-NaOH buffer (○–), 2 μg of CS in the mixture (●–), 2 μg of I^3SO_3-GalCer in the mixture (▲–), and 2 μg of cholesterol in the mixture (○···) at 37°C for 30 min. The reactivity of the enzyme with anti-pancreatic elastase antibody was measured as described in the text.

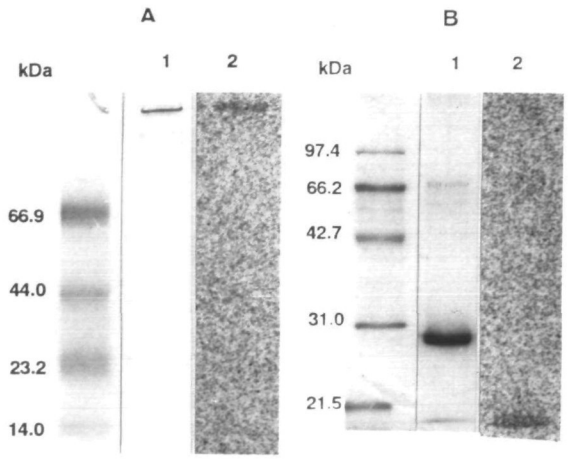

Fig. 7. **Native (A) and SDS-PAGE (B) of pancreatic elastase incubated with ^{35}S-CS.** Pancreatic elastase (5 μg) was incubated with 25 μg of CS containing ^{35}S-CS (1×10^4 cpm), and analyzed by native and SDS-PAGE. Gels were stained with Coomassie brilliant blue (1) and their radioautograms were taken with X-ray film (2).

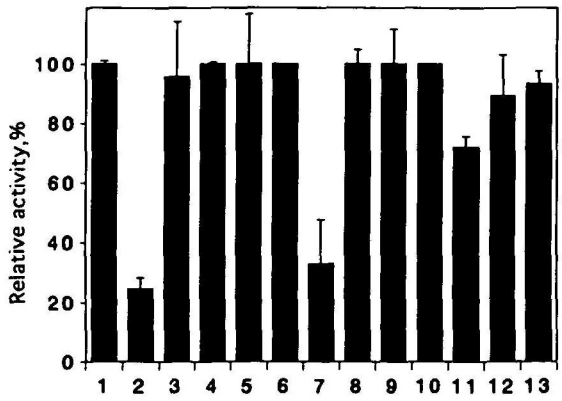

Fig. 8. **Effects of several sulfated derivatives and gangliosides on the activity of porcine pancreatic elastase.** Prior to the addition of substrates, 1 μg of elastase in 90 μl of 50 mM HEPES buffer (pH 7.5) was mixed with 20 nmol of each compound in 10 μl of DMSO, followed by incubation at 37°C for 30 min. The enzyme reaction was initiated by addition of BANP as described in the text. 1, no inhibitor; 2, CS; 3, cholesterol; 4, estrone sulfate; 5, dehydroepiandrosterone sulfate; 6, pregnenolone sulfate; 7, I^3SO_3-GalCer; 8, GalCer; 9, LacCer; 10, $II^3NA\alpha$-LacCer; 11, sodium dodecyl sulfate; 12, sodium taurocholate; 13, Na_2SO_4.

inhibited the elastase activity. Thus, sulfate groups conjugated with hydrophobic structures were found to be essential for inhibition of pancreatic elastase activity.

DISCUSSION

In this study, CS and I^3SO_3-GalCer were shown to be common constituents in the epithelia of mammalian digestive tracts, although their concentrations differed among the mammalian species examined. Under the conditions used for the extraction of lipids, steroid sulfates could not be detected in the final preparation, probably due to their removal in the dialysis step, while the loss of CS was negligible throughout the procedure, as shown by a preliminary experiment using radiolabeled CS. CS was the only sulfated lipid found in the esophageal epithelium, and I^3SO_3-GalCer, together with CS, was detected in the epithelia of the digestive tract distal to the esophagus. In the rat digestive tract, the concentration of I^3SO_3-GalCer was lower than in man, pig, and rabbit, but this was compensated for by a higher concentration of CS, which maintained the level of sulfated lipids at above 0.05 μmol/g dry weight. Among the different regions of the digestive tracts, the concentration of sulfated lipids was particularly high in the duodenal, jejunal, and ileal epithelia, where elastase is converted to the active form for digestion of dietary proteins, and no sulfated lipids were detected in the pancreatic duct, where inactive proelastase is transferred to the duodenum. In support of the hypothesis that sulfated lipids protect the mucosa from injury by protease digestion, they were shown to be localized in the epithelial layer. An immunohistochemical study with monoclonal anti-I^3SO_3-GalCer antibody also demonstrated the localization of I^3SO_3-GalCer in the epithelial cell lining of rabbit and human gastric mucosas (*21*). This selective presence of sulfated lipids in the epithelial layer stands in contrast to the distribution of sialic acid-derived negatively-charged lipids in both the epithelial layer and tunica muscularis. According to previous observations, I^3SO_3-GalCer in the basolateral side of epithelial cells is expected to bind to laminin in the basement membrane to form the epithelial and glandular structures (*22*), and in the apical side it confers acid resistance on the gastric epithelial cells (*23*) and acts as a receptor for *Helicobacter pylori* (*24*) and selectin (*25*). CS has been shown to be distributed in the squamous epithelia of the esophagus and trachea (*26*), the epidermis and its related structures, such as hair and nails, and in the epithelia of the digestive tract of mammals (*20*), and has been shown to activate the η, ε, and ζ forms of protein kinase C, which are involved in squamous epithelial differentiation (*27, 28*). These findings indicate that sulfated lipids have the potential to interact with functional proteins and to modulate their activities for the expression of tissue-characteristic functions. The results obtained in this study also provide evidence for the modulation of the pancreatic enzyme, elastase. Since we also found that CS and I^3SO_3-GalCer inhibit the activity of pancreatic trypsin and chymotrypsin (*15*), the major role of sulfated lipids in the epithelia of mammalian digestive tracts may be to regulate various digestive enzymes by suppressing their activities. The mechanism of inhibition of the enzyme activity by CS was concluded to be different from that of the trypsin inhibitor, which interacts with trypsin in a molar ratio of 1:1 (*29*). The molar ratio of CS to elastase when the enzyme activity was inhibited to 50% of the maximum level was 6:1, and the K_m was not altered after treatment with CS, suggesting that the inhibition does not occur by specific interaction of CS with the active site essential for the enzyme activity. The fact that sulfated lipids, including CS and I^3SO_3-GalCer, form stable complexes with long chain bases under neutral and weakly acidic conditions (*30*) suggests that the vicinal hydroxy and amino groups in elastase and their neighboring hydrophobic region, which is similar in structure to the hydrophobic regions in long chain bases, are involved in the interaction between the enzyme and sulfated lipids. Since there is no structural similarity between cholesterol and GalCer, the sulfate group and the hydrophobic side chain were concluded to be the requisite

structures for the inhibition of elastase. The failure of steroid sulfates to inhibit elastase activity may be due to their low hydrophobicity, which makes it difficult for them to bind with the elastase. The weak inhibition of elastase activity by SDS was also thought to be due to its low hydrophobicity. However, *P. aeruginosa* elastase is more hydrophobic than pancreatic elastase and, therefore, a hydrophobic-hydrophobic interaction alone is not sufficient to explain the inhibition of elastase activity by sulfated lipids (*31, 32*). Since CS-treated elastase lost its ability to react with anti-elastase antibody and occupied a position corresponding to a higher molecular weight than that of the native elastase upon native PAGE, masking or a conformational change of the active site of the enzyme caused by its interaction with sulfated lipids probably resulted in the inhibition of the elastase activity. Sulfated lipids bound to elastase could not be separated from the enzyme by gel permeation chromatography using a TSK-3000 column under neutral or basic conditions (Ito *et al.*, unpublished observation), or by native PAGE, suggesting that sulfated lipids are tightly bound to elastase, causing irreversible inhibition of its activity. Thus, the activities of several proteins with structures accessible to sulfated lipids should be modified negatively or positively: the negatively regulated proteins are trypsin, chymotrypsin, pronase (*15*), acrosin (*33*), 3-hydroxy-3-methylglutaryl CoA reductase (*34*), and phosphatidyl inositol-3-kinase (*35*); and the positively regulated proteins are protein kinase C η, ε, and ζ (*27, 28*), factor XII and prekallikrein (*36*). The distribution patterns of these proteins are identical to those of sulfated lipids, suggesting that natural detergents such as sulfated lipids act as regulators of the activities of bioactive proteins. Proteins whose activities are modified by sulfated lipids are being further studied in our laboratory to elucidate the mechanism of modification and the functional significance of sulfated lipids.

REFERENCES

1. Gertler, A. and Birk, Y. (1970) Isolation and characterization of porcine proelastase. *Eur. J. Biochem.* **12**, 170-176
2. Shotton, D.M. (1970) Elastase. *Methods Enzymol.* **19**, 113-140
3. Stroud, R.M. (1974) A family of protein-cutting proteins. *Sci. Am.* **231**, 74-88
4. Schuster, A., Hansen, G., Zubrod-Eichert, C., and Wahn, V. (1996) Effects of native and oxidation-resistant secretory leukoprotease inhibitor on crystic fibrosis sputum: inhibition of mentrophil elastase activity and of sputum-induced secretive from porcin tracheal submucosal glands. *Pediatr. Res.* **40**, 732-737
5. Dernek, S., Sevin, B., Aslan, R., Kaklillaya, I., Atalay, C., and Kural, T. (1996) Effects of aprotinin on plasma levels of neutrophil elastase and postoperative blood loss in cardiac surgery. *Cardiovascular Surg.* **4**, 515-519
6. Ryu, S.E., Choi, H.J., Kwon, K.S., Lee, K.N., and Yu, M.H. (1996) The native strains in the hydrophobic core and flexible reactive loop of a serine protease inhibitor: Crystal structure of an uncleaved alpha-1-antitrypsin at 2.7 Å. *Structure* **4**, 1182-1192
7. Cook, L., Burdon, J.G., Brenton, S., Knight, K.R., and Janus, E.D. (1996) Kinetic characterisation of alpha-1-antitrypsin F as an inhibitor of human neutrophil elastase. *Pathology* **28**, 242-247
8. Paterson, T. and Moore, S. (1996) The expression and characterization of five recombinant murine alpha-1-protease inhibitor proteins. *Biochem. Biophys. Res. Commun.* **219**, 64-69
9. Cavarra, E., Martorana, P.A., Gambelli, F., Do Santi, M., van Even, P., and Lungaralla, G. (1996) Neutrophil recruitment into the lungs is associated with increased lung elastase burden, decreased lung elastin, and emphysema in alpha-1-proteinase inhibitor-deficient mice. *Lab. Invest.* **75**, 273-280
10. Mellet, P., Michels, B., and Bieth, J.G. (1996) Heat-induced conversion of ovalbumin into a proteinase inhibitor. *J. Biol. Chem.* **271**, 30311-30314
11. Sarfati, I., Lopes, D., Murphy, E.A., Rao, S., and Cohen, J.R. (1996) Inhibition by protease inhibitors of chemotaxis induced by elastin-derived peptide. *J.Surg. Res.* **61**, 84-88
12. Betsuyaku, T., Nishimura, M., Yoshioka, A., Takeyabu, K., Miyamoto, K., and Kawakami, Y. (1996) Elastin-derived peptides and neutrophil elastase in bronchoalveolar lavage fluid. *Am. J. Res. Crit. Med.* **154**, 720-724
13. Vogelmeier, C., Gillissen, A., and Buhl, R. (1996) Use of secretory leukoprotease inhibitor to augment lung antineutrophil elastase activity. *Chest* **110**, 261-266
14. Okajima, K., Inoue, M., Koga, S., Okabe, H., and Takatsuki, K. (1990) Plasma polymorphonuclear leukocyte elastase-alpha-1-proteinase complex in patients with gastric ulcer. *Clin. Chim. Acta* **189**, 237-241
15. Iwamori, M., Iwamori, Y., and Ito, N. (1997) Sulfated lipids as inhibitors of pancreatic trypsin and chymotrypsin in epithelium of the mammalian digestive tract. *Biochem. Biophys. Res. Commun.* **237**, 262-265
16. Momoeda, M., Cui, Y., Sawada, Y., Taketani, Y., Mizuno, M., and Iwamori, M. (1994) Pseudopregnancy-dependent accumulation of cholesterol sulfate due to up-regulation of cholesterol sulfotransferase and concurrent down-regulation of cholesterol sulfate sulfatase in the uterine endometria of rabbits. *J. Biochem.* **116**, 657-662
17. Iwamori, M., Sawada, K., Hara, Y., Nishino, M., Fujisawa, T., Imura, H., and Nagai, Y. (1982) Neutral glycosphingolipids and gangliosides of bovine thyroid. *J. Biochem.* **91**, 1875-1887
18. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254
19. Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1995) *Short Protocols in Molecular Biology*, 10.5-10.25, John Wiley & Sons, New York
20. Cui, Y. and Iwamori, M. (1997) Distribution of cholesterol sulfate and its anabolic and catabolic enzymes in various rabbit tissues. *Lipids* **32**, 599-604
21. Sugano, K., Tai, T., Kawashima, I., Kotani, M., Natomi, H., Kamisago, S., Fukushima, Y., Yazaki, Y., and Iwamori, M. (1995) Localization of sulfatides in the epithelial lining of gastric mucosa: Studies with a monoclonal antibody to sulfatides. *J. Clin. Gastroenterol.* **21**, S98-S103
22. Roberts, D.D., Rao, C.N., Magnani, J.L., Spitalink, S.L., Liotta, L.A., and Ginsburg, V. (1985) Laminin binds specifically to sulfated glycolipids. *Proc. Natl. Acad. Sci. USA* **82**, 1306-1310
23. Natomi, H., Sugano, K., Takaku, F., and Iwamori, M. (1990) Glycosphingolipid composition of the gastric mucosa. *J. Clin. Gastroenterol.* **12**, 52-57
24. Saitoh, T., Natomi, H., Zhao, W., Okuzumi, K., Sugano, K., Iwamori, M., and Nagai, Y. (1991) Identification of glycolipid receptors for *Helicobacter pylori* by TLC-immunostaining. *FEBS Lett.* **282**, 385-387
25. Bengtsson, T., Grenegard, M., Olsson, A., Sjogren, F., Stendahl, O., and Zalavary, S. (1996) Sulfatide-induced L-selectin activation generates intracellular oxygen radicals in human neutrophils: modulation by extracellular adenosine. *Biochim. Biophys. Acta* **1313**, 119-129
26. Rearick, J.I., Albro, P.W., and Jetten, A.M. (1987) Increase in cholesterol sulfotransferase activity during in vitro squamous differentiation of rabbit tracheal epithelial cells and its inhibition by retinoic acid. *J. Biol. Chem.* **262**, 13069-13074
27. Ikuta, T., Chida, K., Tajima, O., Matsuura, Y., Iwamori, M., Ueda, Y., Mizuno, K., Ohno, S., and Kuroki, T. (1994) Cholesterol sulfate: a novel second messenger for the η isoform of protein kinases C-mediating squamous differentiation. *Cell Growth Differ.* **5**, 943-947

28. Denning, M.F., Kazanietz, M.G., Blumberg, P.M., and Yuspa, S.H. (1995) Cholesterol sulfate activates multiple protein kinase C isoenzymes and induces granular cell differentiation in cultured murine keratinocytes. *Cell Growth Differ.* **6**, 1619-1626
29. Kortemme, T., Hollekker, M., Kemmink, J., and Creighton, T.E. (1996) Comparison of the (30-51, 14-38) two-disulphide folding intermediates of the homologous proteins dendrotoxin K and bovine pancreatic trypsin inhibitor by two-dimensional ^{1}H nuclear magnetic resonance. *J. Mol. Biol.* **257**, 188-198
30. Downing, D.T., Dose, R.W., and Abraham, W. (1993) Interaction between sphingosine and cholesteryl sulfate in epidermal lipids. *J. Lipid Res.* **34**, 563-569
31. Shirasu, Y., Yoshida, H., Mikayama, T., Matsuki, S., Tanaka, J., and Ikenaga, H. (1986) Isolation and expression in *Escherichia coli* of a cDNA clone encoding porcine pancreatic elastase. *J. Biochem.* **99**, 1707-1712
32. Fukushima, J., Yamamoto, S., Morihara, K., Atsumi, Y., Takeuchi, H., Kawamoto, S., and Okuda, K. (1989) Structural gene and complete amino acid sequence of *Pseudomonas aeruginosa* IFO 3455 elastase. *J. Bacteriol.* **171**, 1698-1704
33. Burck, P.J. and Zimmerman, R.E. (1980) The inhibition of acrosin by sterol sulphates. *J. Reprod. Fert.* **58**, 121-125
34. Ponec, M. and Williams, M.L. (1986) Cholesterol sulfate uptake and outflex in cultured human keratinocytes. *Arch. Dermatol. Res.* **279**, 32-36
35. Woscholski, R., Kodaki, T., Palmer, R.H., Waterfield, M.D., and Parker, P.J. (1995) Modulation of the substrate specificity of the mammalian phosphatidylinositol 3-kinase by cholesterol sulfate and sulfatide. *Biochemistry* **34**, 11489-11493
36. Shimada, T., Kato, H., Iwanaga, S., Iwamori, M., and Nagai, Y. (1985) Activation of factor XII and prekallikrein with cholesterol sulfate. *Thromb. Res.* **38**, 21-31