Nasal Membrane and Intracellular Protein and Enzyme Release by Bile Salts and Bile Salt–Fatty Acid Mixed Micelles: Correlation with Facilitated Drug Transport

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The effects of four bile salts, one fusidate derivative, and one mixed micellar formulation of bile salt–fatty acid combination on the nasal mucosal protein and enzyme release have been investigated in rats using an *in situ* nasal perfusion technique. Deoxycholate (NaDC) was found to possess the maximum protein solubilizing activity, followed by taurodihydrofusidate (STDHF), cholate, glycocholate (NaGC), and taurocholate (NaTC) in a descending order. The difference in protein solubilization of NaDC and NaGC was further characterized by the release of 5′-nucleotidase (5′-ND), a membrane-bound enzyme, and lactate dehydrogenase (LDH), an intracellular enzyme, in the perfusate. While both NaDC and NaGC caused comparable 5′-ND release from nasal membrane, intracellular LDH release was significantly higher with NaDC. The greater protein and LDH solubilizing effects of NaDC corresponded well with its faster rate of disappearance from the nasal perfusate. Therefore, the dihydroxy bile salt NaDC tends to cause intracellular damage and cell lysis, whereas the trihydroxy bile salt NaGC appears to produce primarily mucosal membrane perturbations. Linoleic acid in the form of soluble mixed micelles with glycocholate caused a further increase in nasal protein release. However, the rate and extent of nasal membrane protein release by the mixed micelles composed of 15 m*M* glycocholate and 5 m*M* linoleic acid were significantly lower than those caused by either deoxyholate or STDHF at the same concentrations. Nasal absorption of acyclovir, a nonabsorbable hydrophilic model antiviral agent, was found to be enhanced in the presence of conjugated trihydroxy bile salts and bile salt–fatty acid mixed micelles. A nonlinear correlation exists between first-order nasal absorption rate constant and nasal protein release rate.

KEY WORDS: absorption enhancement; acyclovir; bile salts; fusidate derivative; lactate dehydrogenase; linoleic acid; mixed micelles; nasal absorption; 5′-nucleotidase; protein release.

INTRODUCTION

Systemic delivery of therapeutic agents through nasal administration has several potential advantages, including ease of administration, patient acceptance, avoidance of first-pass hepatic metabolism, rapid absorption, and high systemic availability. Polar and hydrophobic drug molecules ranging in size from small metal ions to large proteins have been investigated. The rate and extent of nasal drug absorption depend on the aqueous to lipid partition coefficient, pK_a, molecular weight, perfusion rate, instilled solution pH, initial drug concentration, and design of dosage form (1–4).

Hydrophilic and high molecular weight compounds exhibit low and variable nasal absorption. For instance, only a negligible amount of insulin was found to reach the systemic circulation without the presence of nasal absorption enhancers (5). In order to facilitate nasal absorption of compounds with molecular weights >10 kD, such as pharmacologically active peptide hormones and protein drugs, absorption promoters need to be developed. These enhancers belong to various chemical classes including anionic and cationic surfactants (6), bile salts (5,7), bile salt–fatty acid mixed micelles (7), fusidic acid derivatives (8–10), medium-chain fatty acids (11), and cyclodextrin derivatives (12). The mechanisms by which enhancers increase the nasal absorption are speculative; several hypotheses have been proposed, i.e., alteration of biological membrane structure, inhibition of protease activity, and dissociation of molecular aggregates through micellar solubilization. It is, therefore, an objective of this investigation to characterize some of these nasal absorption enhancers in order to understand the underlying mechanisms and to design safe enhancing agents that result in minimal and acceptable membrane damage.

Bile salts are known to solubilize membrane proteins, phospholipids, and enzymes (13–15). The solubilization of membrane components by enhancers may be related to the compound's ability to overcome the nasal membrane barrier resistance. In this study, the release of a membrane protein and a cytoplasmic protein, namely, 5′-nucleotidase (5′-ND) and lactate dehydrogenase (LDH), respectively, from the nasal cavity were measured in the presence of bile salts, linoleic acid, and a dihydrofusidate derivative. Further, the ability of these agents to enhance the nasal transport of a nasally nonabsorbable hydrophilic compound, acyclovir, was compared with the protein release rate. Finally, the rate of bile salt loss from the perfusion medium was determined to understand the relationship between bile salt uptake into the mucosal epithelium and nasal transport enhancement.

MATERIALS AND METHODS

Chemicals

Acyclovir [9-(2-hydroxyethoxymethyl)guanine] was a gift from Burroughs Wellcome Company (Research Triangle Park, NC). Sodium deoxycholate (NaDC) and heptanesulfonic acid, sodium salt, were obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI). Sodium taurodihydrofusidate (STDHF) was kindly donated by Leo Pharmaceutical Products (Ballerup, Denmark). Sodium cholate, sodium glycocholate (NaGC), sodium taurocholate (NaTC), linoleic acid, lactate dehydrogenase (LDH), and 5′-nucleotidase (5′-ND) analytical kits were supplied by Sigma Chemical Company (St. Louis, MO). Other reagents were of analytical grade and were used as received.

Preparation of Micellar and Mixed Micellar Solutions

Bile salt or STDHF was added to isotonic phosphate buffer, pH 7.4, consisting of 0.014 *M* KH_2PO_4, 0.057 *M*

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Na_2HPO_4, and 0.070 *M* NaCl and sonicated for 5 min at room temperature with a Branson sonicator (Model 3200, Branson Co., Shelton, CT). In the case of mixed micellar solutions, linoleic acid was added after the bile salt was dissolved. All solutions were freshly prepared and used immediately.

In Situ Nasal Perfusion Method

The rat *in situ* nasal perfusion technique developed by Hirai *et al.* (16) and Huang *et al.* (2) was used in this investigation due to the fairly simple nature of the experimental setup and good reproducibility of the method. Male Sprague–Dawley rats weighing 250 to 350 g were fasted for about 14 to 18 hr prior to use but water was allowed ad libitum. The rats were anesthetized by an intraperitoneal injection of 0.1 ml/100 g body wt of a ketamine (90 mg/ml) and xylazine (10 mg/ml) mixture, followed by an additional 0.1 ml/rat every 30 to 45 min to maintain the anesthetic state. After an incision was made in the neck, the trachea was cannulated with a polyethylene tube (PE-200, Intramedic, Clay Adams, NY) to maintain respiration. Another PE-200 tube was inserted through the esophagus toward the posterior part of the nasal cavity and ligated. The passage of the nasopalatine tract was sealed with an adhesive agent (Instant Jet, Cal Goldberg Models Inc., Chicago, IL) to prevent drainage of the solution from the nasal cavity to the mouth. The cannula served to deliver the solution to the nasal cavity. The perfusion medium was circulated by means of a peristaltic pump at a flow rate of 2 ml/min and recollected into a reservoir. The temperature of the reservoir was maintained at 37 ± 0.5°C during the course of an experiment. A constant perfusate volume of 5 ml was maintained throughout with constant stirring and an aliquot (100 μl) was sampled every 15 min for 1.5 hr.

Analytical Procedures

The protein contents of the perfusate at various sampling points were measured by the method of Lowry *et al.* (17). Bovine serum albumin (BSA) was used as the standard. The presence of bile salts, STDHF, linoleic acid, and acyclovir did not interfere with the assay. A standard curve was constructed with the samples in order to minimize errors. Absorbance at 750 nm was measured with a Beckman DU-7 UV/VIS spectrophotometer (Irvine, CA).

The activity of a nasal membrane-bound enzyme, 5′-nucleotidase (EC 3.1.3.5), in the perfusate was assayed according to a kinetic method reported by Arkesteijn (18). The nasal epithelial intracellular enzyme activity of lactate dehydrogenase (EC 1.1.1.27) was determined by the method of Cabaud and Wroblewski (19).

The concentration of acyclovir remaining in the nasal perfusate was determined by a modified HPLC method of Land and Bye (20). Aliquots of 100-μl samples were withdrawn periodically from the reservoir and immediately mixed with equal volumes of acetonitrile containing 20 μg/ml thymine as the internal standard. The samples were vigorously vortexed for 30 sec and centrifuged at 10,000 rpm for 15 min in order to precipitate any proteins prior to sample injection onto the HPLC column. The HPLC system was equipped with a Waters Model 510 solvent delivery system, a Rheodyne injector, a Waters Lambda-Max Model 481 multiwavelength UV detector, and a Fisher Recordall Series 5000 strip-chart recorder. Samples (10 μl) were injected onto an Alltech Econosil 10-μm spherical C_{18} reversed-phase column (250 × 4.6 mm) at ambient temperature. The mobile phase was composed of 2% acetonitrile (v/v) in pH 5.0 ammonium acetate buffer containing 1.0 m*M* heptanesulfonic acid, sodium salt, and was pumped at a rate of 2 ml/min. The wavelength for detection was set at 254 nm. Acyclovir was found to be stable in rat nasal perfusate.

The change in deoxycholate and glycocholate concentration in the perfusate as a function of time was determined by a HPLC method reported by Armstrong and Carey (21) with slight modifications. The mobile phase consisted of 75% (v/v) acetonitrile:25% (v/v) water buffered with 5 m*M* KH_2PO_4/H_3PO_4 (pH 5.4) for deoxycholate and 30% (v/v) acetonitrile:70% (v/v) water buffered with 5 m*M* KH_2PO_4/H_3PO_4 for glycocholate to render adequate retention. The wavelength for detection was set at 210 nm. In order to eliminate interference from solubilized proteins, the sampled perfusate was premixed with 10 vol of ice-cold acetonitrile and centrifuged subsequently for 10 min at 10,000 rpm prior to injection onto the HPLC.

RESULTS AND DISCUSSION

Effects of Different Bile Salts and STDHF on Rat Nasal Protein Release

After incorporating deoxycholate, cholate, glycocholate, and taurocholate individually into the nasal perfusion medium, perfusion experiments were carried out for 90 min. The initial bile salt concentration was kept constant at 15 m*M*, which was optimal for enhancement of [D-Arg^2]-kyotorphin nasal absorption (22). Deoxycholate, a dihydroxy unconjugated bile salt, caused the greatest protein release from the nasal cavity, while cholate, glycocholate, and taurocholate, in descending order, exhibited significantly less release (Fig. 1). At 90 min the total protein released by sodium deoxycholate was 3.0, 5.4, and 6.4 times that caused by sodium cholate, sodium glycocholate, and sodium taurocholate, respectively. Further, cholate (trihydroxy bile salt)

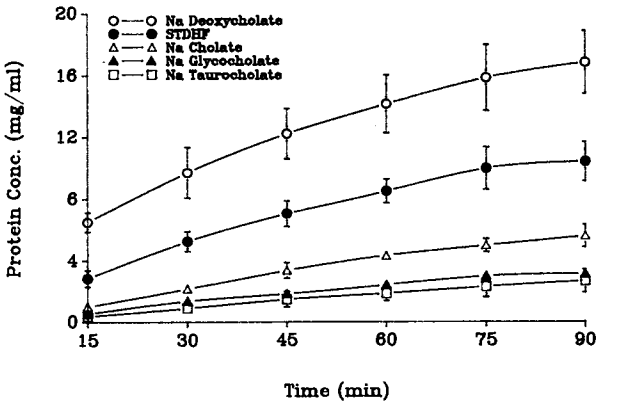

Fig. 1. Protein concentrations in the rat nasal perfusates containing various bile salts at 15 m*M* concentration. Data are means ± SD (n = 3). No significant differences were found between NaGC and NaTC by one-way ANOVA.

caused significantly higher nasal protein release than the corresponding conjugated trihydroxy bile salts. Although the average protein concentration in the presence of 15 m*M* taurocholate was slightly lower than that of glycocholate, no significant differences were found between them at the same respective sampling points. The results are consistent with the *in vivo* intranasal absorption data of insulin in human volunteers reported by Gordon and co-workers (7). The authors found that nasal absorption of insulin correlates well with the hydrophobicity of unconjugated bile salts, with the rank order being deoxycholate > chenodeoxycholate > cholate > ursodeoxycholate. Recently, the intranasal absorption of gentamicin in the presence of bile salts was reported by Duchateau *et al.* (23). Without an enhancer, gentamicin was not absorbed across the nasal mucosa, while the bioavailability increased with the increase in the hydrophobicity of the trihydroxy bile salts (cholate > glycocholate > taurocholate).

In addition to the bioavailability data, ciliotoxicity studies were also reported (23). Deoxycholate was found to be extremely ciliotoxic such that ciliary arrest occurred within 1 min even at a concentration of 5 m*M*. The ciliotoxicity of the bile salts increased with increasing hydrophobicity, with the dihydroxy bile salts exhibiting more toxicity than the trihydroxy bile salts. These results closely concur with our protein release data shown in Fig. 1. Therefore protein release data may serve as a toxicity indicator of various nasal absorption enhancers.

The solubilizing effects of bile salts on human erythrocytes and rat liver plasma membrane have been studied by Coleman and co-workers (13–15). The membrane-damaging properties of the bile salts were found in the order deoxycholate > cholate > glycocholate and taurocholate. Simultaneous solubilization of membrane protein and phospholipid was found with all the bile salts studied. However, very limited information concerning the release of membrane-bound and intracellular enzymes from the nasal cavity in the presence of surfactants is available.

Sodium taurodihydrofusidate (STDHF) was shown to have a high efficacy in enhancing the intranasal absorption of insulin in both animal experiments and human clinical trials (8,9). STDHF was 5 to 10 times less lytic than deoxycholate, and at least 100-fold less lytic than nonionic surfactants. In order to compare the protein release properties of STDHF with those of the bile salts, the protein release profile of STDHF is plotted in Fig. 1 as well. In agreement with the hemolytic results of Longenecker *et al.* (8), STDHF indeed caused lower protein release compared to deoxycholate (38% lower at 90 min). Nevertheless, it still caused considerably higher amount of protein release than conjugated trihydroxy bile salts from the rat nasal cavity. Therefore, the selection of STDHF as a safe nasal enhancer deserves further investigation.

Nasal Membrane-Bound and Intracellular Enzyme Activities in the Perfusate

Little information is available on nasal enzyme activity and its role in drug absorption and degradation. Various enzymes were shown to exist in nasal secretions (24). As bile salts caused considerable total protein release, one needs to determine specific enzyme activities in the perfusate. Two marker enzymes, i.e., membrane-bound 5′-nucleotidase (5′-ND) and intracellular lactate dehydrogenase (LDH), were measured as a function of perfusion time. Figure 2 illustrates the activity of 5′-ND in the nasal perfusate in the presence of 15 m*M* NaDC, 15 m*M* NaGC, and isotonic phosphate buffer. Isotonic phosphate buffer (PBS) wash did not cause any significant release of 5′-ND, with the activity being 2.2 ± 1.7 U/L at 90 min. In contrast, the presence of 15 m*M* NaDC and NaGC caused significant release ($P < 0.05$) of 5′-ND, with the activities being 114.7 ± 19.4 and 87.7 ± 12.0 U/L at 90 min, respectively. Besides, the profiles of 5′-ND release in the presence of bile salts appear to indicate a saturable process, approaching a plateau level after 30 to 60 min. When compared statistically, one-way ANOVA failed to reveal any significant difference between the two bile salts at the same time points, thus indicating comparable effects.

Figure 3 demonstrates the activities of LDH as a function of perfusion time. Once again the phosphate buffer washing did not cause any significant release of LDH, producing an average activity of 33.0 ± 18.0 U/ml at 90 min. NaGC (15 m*M*) caused significantly higher LDH release ($P < 0.05$) than that of PBS (1.04 ± 0.40 KU/ml at 90 min). However, NaDC resulted in the highest LDH release, reaching 12.60 ± 3.17 KU/ml at 90 min. This drastic difference in LDH release indicates the varying capabilities of bile salts in creating leaky biological membranes.

Since both NaDC and NaGC resulted in similar membrane-bound enzyme activity in the nasal perfusate, the different intracellular enzyme release accounts for the difference in total nasal protein release between NaDC and NaGC.

Since different bile salts exhibit dramatic differences in releasing intracellular proteins, the severity of membrane damage may be attributed to their ability to penetrate the nasal membrane. Therefore, the disappearance of NaDC and NaGC from the perfusate was measured (Fig. 4). Indeed, the rate of loss of NaDC from nasal perfusate is faster than that of NaGC. When fitted by apparent first-order kinetics, average disappearance rate constants of $6.582 \times 10^{-3} \pm 1.266 \times 10^{-3}$ min^{-1} (mean ± SD; $n = 3$) and $3.410 \times 10^{-3} \pm 1.040 \times 10^{-3}$ min^{-1} ($n = 3$) were obtained for NaDC and NaGC, respectively. One-way ANOVA comparison revealed a significant difference at a confidence level of 95%.

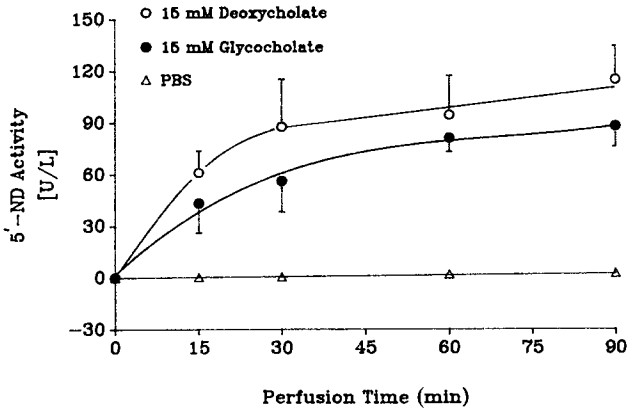

Fig. 2. Release of 5′-nucleotidase from the rat nasal cavity as a function of perfusion time. Data are means ± SD ($n = 3$).

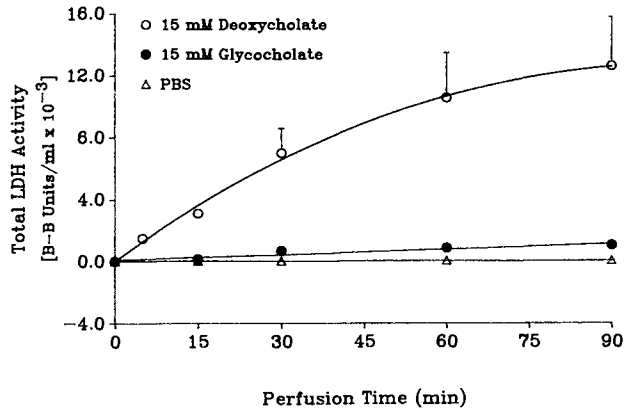

Fig. 3. Release of lactate dehydrogenase from the rat nasal cavity as a function of perfusion time. Data are means ± SD (n = 3).

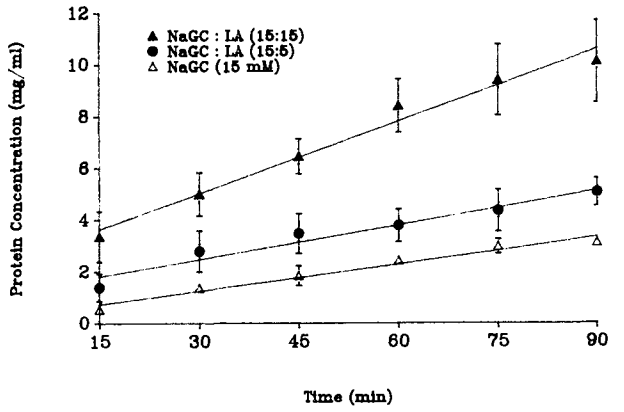

Fig. 5. Protein concentrations in rat nasal perfusates containing 15 mM sodium glycocholate and 0, 5, and 15 mM linoleic acid. Values represent means ± SD (n = 3).

Effect of Varying Linoleic Acid Concentrations on Nasal Protein Release

The promoting effect of bile salt–fatty acid mixed micelles on nasal absorption of [D-Arg^2]kyotorphin appeared to be synergistic, i.e., greater than that produced by bile salt or fatty acid alone (22). Therefore, we wanted to investigate the effect of incorporation of linoleic acid in 15 mM sodium glycocholate micellar solution on nasal protein release. The addition of 5 and 15 mM linoleic acid in the form of mixed micelles caused significantly higher protein release (Fig. 5). The average linear protein release rates are $0.0334 \pm 2.05 \times 10^{-3}$, $0.0460 \pm 2.91 \times 10^{-3}$, and 0.0914 ± 0.0130 mg/ml/min (n = 3) for 15 mM NaGC containing 0.5, and 15 mM linoleic acid, respectively. Further, the average protein release rate correlates linearly with the concentration of linoleic acid in the mixed micellar solution, with a correlation coefficient of 0.992. This correlation strongly indicates that the solubilized form of linoleic acid facilitates the interaction of mixed micelles with nasal membrane components, resulting in increased membrane fluidity and permeability. The enhanced permeability caused by fatty acids on intestinal epithelium was attributed to the disorder in the hydrophobic region of the membrane interior and interaction with the polar head groups of phospholipids (25). A differential scanning calorimetric study (26) also suggested that fatty acids can modify phase transition within membrane structure by interacting with the lipid structure and disrupting its ordered state.

Effect of Varying Sodium Glycocholate Concentrations on Nasal Protein Release and Acyclovir Absorption

Since conjugated trihydroxy bile salts resulted in the least amount of nasal protein release, sodium glycocholate (NaGC) was selected as a model compound to study further the effect of bile salt concentration on the rat nasal protein release and acyclovir absorption. The concentration of NaGC incorporated into the perfusion media ranged from 0 to 35 mM. Protein release increased at a linear rate with an increase in initial NaGC concentration in the perfusate. The calculated rates of total protein release are plotted as a function of NaGC concentration (Fig. 6).

Acyclovir, a purine nucleoside antiviral agent, was reported to exhibit poor and variable absorption from the GI tract following oral administration. The oral bioavailability was found to be low (20–40%) and species dependent (27). No cutaneous penetration of acyclovir was observed without adjuvants, while the presence of oleic acid in a propylene glycol base significantly increased skin transport (28). The poor absorption characteristics of acyclovir are due partly to

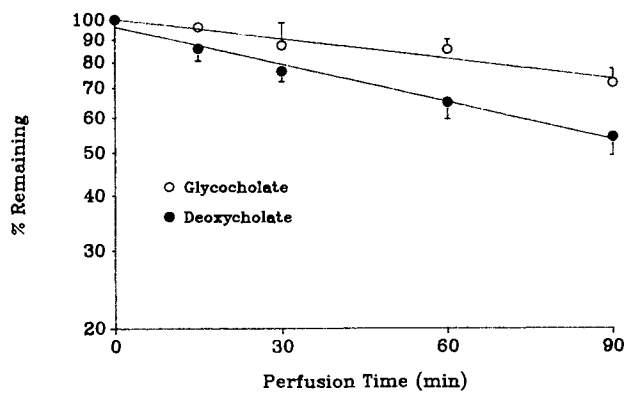

Fig. 4. Percentage of bile salt remaining in the perfusate as a function of time. Data represent means ± SD (n = 3).

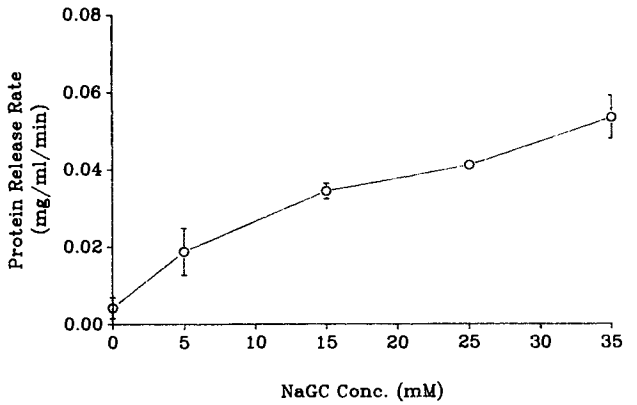

Fig. 6. The rate of protein release plotted as a function of sodium glycocholate concentration. Values represent means ± SD (n = 3).

its poor partition coefficient. Because of its limited membrane permeability and excellent stability in the nasal perfusion medium, this compound was selected as a model drug to study the enhancing effect of bile salts and mixed micelles.

The initial concentration of acyclovir in the perfusion medium was maintained at 50 μ*M*. Nasal perfusion studies were first conducted without the presence of adjuvants, no measurable loss of acyclovir from the perfusate was observed. When NaGC was incorporated in the perfusate at concentrations ranging from 5 to 35 m*M*, the rate and extent of absorption increased. Since *in vitro* incubation of acyclovir with nasal washings revealed no change in its concentration for at least 10 hr, the loss of the drug from the perfusate therefore indicates its absorption across the nasal mucosa. Loss of acyclovir from the perfusate followed first-order kinetics, indicating absorption by passive diffusion over 90 min (not shown). The first-order absorption constants were calculated and plotted against NaGC concentration as shown in Fig. 7. The nasal absorption rate constant of acyclovir increases almost linearly in the presence of 5 to 25 m*M* NaGC, leveling off at 35 m*M* NaGC. By comparing Figs. 6 and 7, it appears that protein release and enhanced nasal acyclovir absorption are correlated.

Correlation Between Acyclovir Nasal Absorption and Protein Release in the Presence of NaGC, NaTC, and NaGC–Linoleic Acid Mixed Micelles

As shown in Figs. 6 and 7, increments in NaGC concentration resulted in progressively increased amounts of protein release, with simultaneous enhancement of acyclovir nasal absorption. To compare drug absorption enhancement with protein release, NaGC (5, 15, 25, and 35 m*M*), NaTC (15 m*M*), and NaGC–linoleic acid (15:5) mixed micellar solutions each containing 50 μ*M* initial acyclovir were perfused through the rat nasal cavity. The acyclovir absorption rate constants from 20 individual rat experiments were plotted against the respective protein release rates as illustrated in Fig. 8. Below the protein release rate of 0.01 mg/ml/min, the nasal membrane is impermeable to acyclovir molecules. Within the range of 0.01 to 0.05 mg/ml/min, the protein release rate is linearly related to the increase in acyclovir absorption rate constant. Beyond 0.05 mg/ml/min, the nasal absorption of acyclovir reaches a plateau, exhibiting no further absorption enhancement.

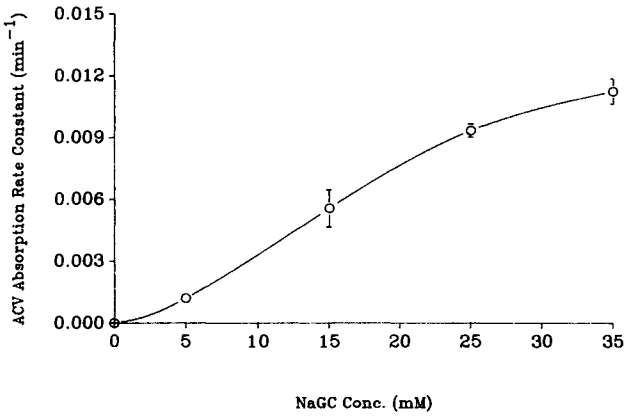

Fig. 7. The first-order acyclovir nasal absorption constants versus sodium glycocholate concentration. Values represent means ± SD (n = 3).

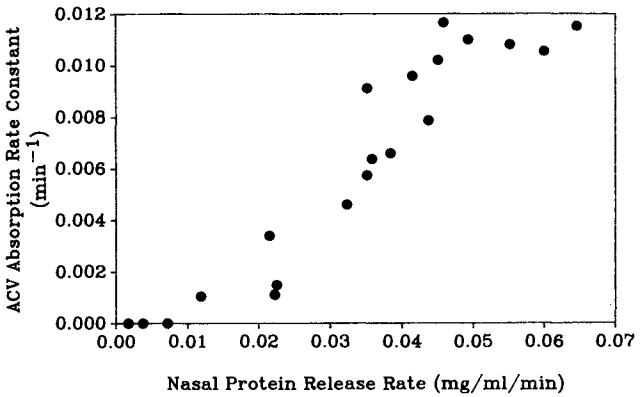

Fig. 8. Correlation between nasal acyclovir absorption rate constants and nasal protein release rates.

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