# Inhibition of lipases by ε-polylysine

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**Abstract Oral administration of ε-polylysine to rats reduced the peak plasma triacylglycerol concentration. In vitro, ε-polylysine and polylysine strongly inhibited the hydrolysis, by either pancreatic lipase or carboxylester lipase, of trioleoylglycerol (TO) emulsified with phosphatidylcholine (PC) and taurocholate. The ε-polylysine concentration required for complete inhibition of pancreatic lipase, 10 μg/ml, is 1,000 times lower than that of BSA required for the same effect. Inhibition requires the presence of bile salt and, unlike inhibition of lipase by other proteins, is not reversed by supramicellar concentrations of bile salt. Inhibition increases with the degree of polylysine polymerization, is independent of lipase concentration, is independent of pH between 5.0 and 9.5, and is accompanied by an inhibition of lipase binding to TO-PC emulsion particles. However, ε-polylysine did not inhibit the hydrolysis by pancreatic lipase of TO emulsions prepared using anionic surfactants, TO hydrolysis catalyzed by lingual lipase, or the hydrolysis of a water-soluble substrate. In the presence of taurocholate, ε-polylysine becomes surface active and adsorbs to TO-PC monomolecular films. These results are consistent with ε-polylysine and taurocholate forming a surface-active complex that binds to emulsion particles, thereby retarding lipase adsorption and triacylglycerol hydrolysis both in vivo and in vitro.**—Tsujita, T., M. Sumiyoshi, T. Takaku, W. E. Momsen, M. E. Lowe, and H. L. Brockman. **Inhibition of lipases by ε-polylysine. *J. Lipid Res.* 2003.** 44: **2278–2286.**

**Supplementary key words** basic peptide • complex • lipid emulsion • lipid monolayer

In mammals, dietary neutral lipid digestion is commonly assumed to be mediated by three main enzymes: preduodenal (lingual or gastric) lipase, carboxylester lipase (cholesterol esterase), and pancreatic lipase (1). Typical substrates for these enzymes are water-insoluble long-chain triacylglycerols. In contrast to their lipid substrates, these lipases are water soluble. Thus, for catalysis to occur, these enzymes must be adsorbed to the lipid surfaces and, therefore, the quality of the surface of substrate lipids is an important factor for lipase activity. Enzymes are sometimes activated or denatured by surface adsorption (2). As a consequence of these properties, amphiphilic substances, acting as emulsifiers, would be expected to influence the lipase reaction rate. It is well known that bile salts and synthetic detergents behave as inhibitors of lipolysis (3, 4). Amphiphilic proteins, such as BSA and β-lactoglobulin, have been shown to inhibit lipase activity toward its triglyceride substrate (5, 6).

Previously, we demonstrated that a basic protein, protamine, strongly inhibited the hydrolysis of trioleoylglycerol (TO) emulsified with phosphatidylcholine (PC) (7). Polylysine, another basic protein, has been suggested as a food antiseptic. Polylysine binds to some proteins, nucleic acids, viruses, or bacteria through electrostatic or hydrophobic interaction and inhibits their functions. Another form of polylysine, ε-polylysine, is synthesized by linking the α-carboxyl groups of lysine with its ε-amino groups. As a consequence of this linkage, it is not hydrolyzed by proteases, such as trypsin, but retains its basic character. Therefore, ε-polylysine is a candidate for an agent that inhibits intestinal lipid absorption while resisting proteolysis. The experiments presented in this report were undertaken to evaluate this possibility and elucidate its mode of action.

## MATERIALS AND METHODS

### Materials

*Reagents.* The enzyme substrates and reagents used were obtained as follows. TO, taurocholate, deoxycholate, colipase, and β-lactoglobulin were from Sigma (St. Louis, MO). PC (from soybean) was from Nippon Shoji (Tokyo, Japan). Phosphatidylethanolamine (PE, from egg), phosphatidylserine (PS, from bovine brain), phosphatidic acid (PA, from egg PC), and PC (from egg)

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Abbreviations: PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SOPC, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; TO, trioleoylglycerol.

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were from Serdary Research Laboratories (London, Canada). ε-Polylysine (average molecular weight 5,000) was from Chisso Co. (Tokyo, Japan) and polylysine (average molecular weight 1,000–4,000) was from Wako Pure Chemical Industries (Osaka, Japan). The defined length polylysine polypeptides were synthesized using a peptide synthesizer (ABI 432A Synergy, Applied Biosystems Japan, Tokyo, Japan) following the manufacturer's recommendations. BSA was from Wako Pure Chemical Industries and was extracted by the method of Chen (8) to remove free fatty acid.

*Proteins.* Pancreatic lipase for emulsion studies was purified from rat pancreas by the procedure of Gidez (9) with some modifications. Activity during purification was monitored using the soybean PC-TO assay described below under "Enzyme activity assays." The purified enzyme (3,200 U/mg protein, at pH 6.8) gave a single band on SDS-PAGE from which its molecular weight was estimated to be 49,000. Carboxylester lipase was purified from porcine pancreas by the procedure of Rudd, Mizuno, and Brockman (10) with some modifications (11). The purified enzyme preparations were found to have specific activities of 700–800 μmol *p*-nitrophenol released/mg protein/min with *p*-nitrophenyl butyrate as the substrate. Lingual lipase fraction was prepared from rat tongues. The entire lingual serous glandular region was homogenized in cold 25 mM potassium phosphate buffer (pH 6.3) containing 0.9% NaCl. The homogenate was centrifuged at 100,000 *g* for 60 min, and the supernatant, which was used as the enzyme solution, was stored at −80°C. An inactive form of human pancreatic lipase (HPL) in which the active site serine was replaced by glycine, HPL(S153G), was constructed by polymerase chain reaction using the Stratagene QuickChange site-directed mutagenesis kit as previously described for colipase mutants (12). The mutant protein was expressed in *Pichia pastoris* and purified as described for native HPL (13).

HPL(S153G) and ε-polylysine were radiolabeled by reductive methylation as described for porcine colipase (14). The ratios of reagents were adjusted to compensate for the different number of free amino groups in the three proteins. Specifically, 37 nmol (1.87 mg) of HPL(S153G) was mixed with 21.2 μmol of $NaBH_3CN$ and 7.5 μCi (144 nmol) of [$^{14}$C]HCHO in 1.0 ml final volume; and 1.2 μmol (6 mg) of ε-polylysine was mixed with 31.5 μmol of $NaBH_3CN$ and 37.9 μCi (678 nmol) of [$^{14}$C]HCHO in 3.8 ml final volume. Dialysis was carried out using small volume Spectra/Por® DispoDialyzers® (Spectrum Laboratories, Inc., Laguna Hills, CA). The resulting specific radioactivities of [$^{14}$C]HPL(S153G) and [$^{14}$C]ε-polylysine were 1.96 μCi/mg (220 dpm/pmol) and 4.1 μCi/mg (45.6 dpm/pmol), respectively.

## Methods

*Enzyme activity assays.* Rat pancreatic lipase activity was determined by measuring the rate of release of oleic acid from TO. A suspension of 90 μmol TO, 12.6 μmol soybean PC, and 9.45 μmol taurocholate in 9 ml 50 mM Tris (pH 7.0) containing 0.1 M NaCl was sonicated for 5 min. The assay system was comprised of the following components in a total volume of 200 μl: 25 μl enzyme solution, 50 μl inhibitor solution, 0.25 μg colipase, 0.5 μmol TO, 0.053 μmol taurocholate (except where noted), 0.07 μmol phospholipid, 10 μmol Tris, and 20 μmol NaCl. Incubation was carried out at pH 7.0 and 37°C for 30 min. The amount of oleic acid produced was determined by the method of Zapf et al. (15) with a slight modification (16). Activities measured using this assay were a direct function of lipase added measured activity but decreased parabolically with added enzyme. As a consequence, in experiments showing substantial enzyme inhibition, the extent of inhibition may be underestimated. Carboxylester lipase activity was determined using the same assay without colipase.

In some experiments, rat pancreatic lipase activity against TO was determined using the assay described above but with the 12.6 μmol of soybean PC replaced by 45 mg of gum arabic. Methyl butyrate-hydrolyzing activity by pancreatic lipase was measured as described previously (17). Cholesterol esterase activity of carboxylester lipase was determined by measuring the rate of oleic acid release from cholesteryl oleate as described previously (7). Lingual lipase activity was assayed using soybean PC-emulsified TO as described above for the pancreatic lipase assay but without added bile salt and using 0.1 M acetate buffer (pH 5.0) 0.1 M NaCl.

*Pancreatic lipase distribution assay.* An antipancreatic lipase antiserum was raised in rabbits using purified rat pancreatic lipase (18). Pancreatic lipase was incubated with the TO-PC-taurocholate assay emulsion containing various amounts of ε-polylysine. After 10 min incubation, the supernatant and lipid layer were separated by centrifugation at 10,000 rpm for 10 min and were suspended in Laemmli sample buffer containing 1% and 20% (w/v) SDS, respectively. An aliquot of each suspension (10 μl) was subjected to SDS-PAGE. For Western blotting, the proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA), which was blocked with 5% (w/v) skimmed milk and incubated with the antibody (19). Immunoreactivity was visualized with alkaline phosphatase-conjugated goat anti-rabbit IgG and attophos (ICN Pharmaceuticals, Inc., Aurora, OH), and the enhanced chemifluorescence intensity was determined using a FluorImager fluorescence imaging analyzer (Amersham Pharmacia Biotech UK Ltd.).

*Oral administration of ε-polylysine.* A suspension of 6 ml corn oil, 80 mg cholic acid, and 2 mg cholesterol oleate in 6 ml water was sonicated for 5 min. Male Wistar King rats, weighing 200–250 g and divided into two groups, were starved overnight, and 1 ml corn oil suspension was administered to each rat via a stomach tube. One group received 1.0 ml of this suspension containing 50 mg of peptide (ε-polylysine or BSA) or free lysine, and the control group received a suspension containing 1.0 ml water. At regular intervals after ε-polylysine administration, blood samples were collected from the tail vein or artery into heparinized microcapillary tubes and centrifuged immediately at 10,000 rpm for 5 min. Plasma triacylglycerol concentrations were determined using Triglyceride E-Test (Wako Pure Chemical Industries).

*Lipid monolayer experiments.* The experimental details for the measurement of the adsorption of proteins to lipid monolayers have been described previously (20, 21). Briefly, a cylindrical Teflon trough (surface area, 20.03 cm$^2$; volume, 17.9 ml) was filled with a buffer consisting of 50 mM Tris and 100 mM NaCl (pH 7.0). Surface pressure was monitored using the Wilhelmy method and temperature was held at 24°C. Lipid films were spread from a hexane-ethanol (95:5; v/v) solution until the desired surface pressure was reached. After allowing the lipid monolayer to stabilize, inorganic [$^{32}$P]phosphate was introduced into the stirred aqueous subphase from a microsyringe through a small port in the side of the circular compartment. One minute later an aliquot of [$^{14}$C]protein solution was added in the same manner. Surface pressure was monitored until the monolayer was collected on one side of a hydrophobic filter paper disk, and the extent of [$^{14}$C]protein adsorption was determined. As described, inorganic [$^{32}$P]phosphate was used to correct the measured [$^{14}$C]protein adsorption for the amount of protein in the aqueous subphase that adheres to the paper (22).

# RESULTS

## Emulsion studies

**Figure 1** shows the time course of the plasma triacylglycerol concentration when corn oil emulsion with or with-

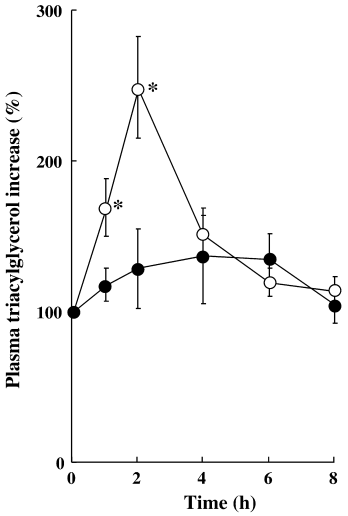


**Fig. 1.** Effect of ε-polylysine on rat plasma triacylglycerol levels after oral administration of lipid emulsion. Lipid emulsion alone (open circle) or lipid emulsion containing ε-polylysine (closed circle) was orally administered. The results are expressed as mean ± SE of eight experiments. * $P < 0.05$.

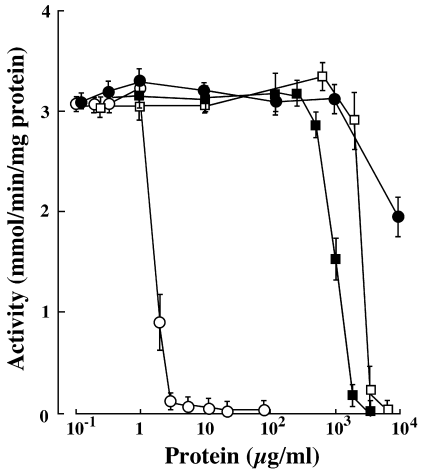


**Fig. 2.** Effect of increasing concentration of proteins on the rate of hydrolysis by rat pancreatic lipase of trioleoylglycerol (TO) emulsified with phosphatidylcholine (PC). Experiments were performed in the presence of 0.265 mM taurocholate and variable concentrations of ε-polylysine (open circle), β-lactoglobulin (closed square), BSA (open square) and lysine (closed circle). Each point represents the mean ± SE of four separate experiments.

out ε-polylysine was administered orally to rats. Two hours after ε-polylysine administration, the plasma triacylglycerol concentration was decreased as compared with controls, but values were comparable after 4 h, 6 h, and 8 h. Clearly, the peak plasma triacylglycerol concentration was reduced by ε-polylysine administration. None of the additives, BSA, free lysine, or peptide, altered plasma triacylglycerol levels (data not shown).

To determine if the effect on triglyceride levels in the serum could be due to impaired triglyceride hydrolysis in the intestine, the hydrolytic activity of rat pancreatic lipase toward TO emulsified with taurocholate (0.265 μM) and soybean PC was determined in the presence of increasing concentrations of ε-polylysine (**Fig. 2**). ε-Polylysine inhibited TO hydrolysis strongly; activity fell by >90% between 0.2 and 0.4 μM (1–2 μg/ml), and hydrolysis was completely inhibited at 2 μM (10 μg/ml). On a weight basis, this concentration is about 1,000 times lower than that of β-lactoglobulin or BSA required to produce the same effect. A similar inhibitory effect was observed using polylysines of differing extent of polymerization (**Fig. 3**). This comparison on a weight concentration basis shows that the higher the extent of polymerization, the more effective is the inhibition. Additionally, free lysine, up to 8,000 μM (1 mg/ml), did not affect TO hydrolysis, and at 80,000 μM (10 mg/ml) it inhibited lipase activity by only 40%. These observations show that neither the absolute concentration of protein nor that of charged free amino groups alone is responsible for the observed inhibition of pancreatic lipase. Rather, the polymer is required.

The inhibitory action of ε-polylysine was determined using three different concentrations of pancreatic lipase over a 4-fold range (data not shown). When normalized to the activity observed in the absence of inhibitor, the extent of lipase inactivation by each ε-polylysine concentration tested was independent of the enzyme concentration. This shows that either the polymer is not acting directly on lipase or that, if it is, the effect is saturated at all lipase concentrations tested. Because of the known sensitivity of lipolysis to the "quality" of the substrate-containing interface, the above results suggested that ε-polylysine might exert its inhibitory effect directly on the interface. To further test this, the effect of 2 μM (10 μg/ml) ε-polylysine on TO hydrolysis by pancreatic lipase was studied using

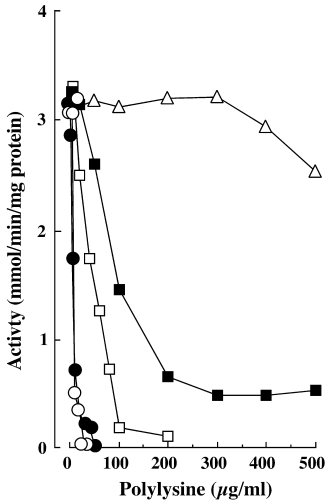


**Fig. 3.** Effect of increasing polylysine length on the rate of hydrolysis by rat pancreatic lipase of TO emulsified with PC. Experiments were performed in the presence of 0.265 mM taurocholate and variable concentrations of ε-polylysine (open circle), polylysine decamer (closed circle), polylysine octamer (open square), polylysine hexamer (closed square), or polylysine tetramer (triangle). Each point represents the mean ± SE of three separate experiments.

TABLE 1. Effect of phospholipids on the trioleoylglycerol-hydrolyzing activity of pancreatic lipase

| Emulsifier | Activity | |
|---|---|---|
| | Control | ε-Polylysine |
| | *μmol/mg/min* | *10 μg/ml* |
| Soybean PC | 441.0 ± 7.36 | 6.1 ± 0.57 |
| Egg PC | 428.4 ± 3.44 | 9.3 ± 0.79 |
| Egg PE | 361.0 ± 3.08 | 36.1 ± 0.90 |
| Bovine brain PS | 442.0 ± 5.35 | 514.5 ± 5.55 |
| Egg PA | 360.0 ± 2.12 | 418.3 ± 6.01 |
| Gum arabic | 342.8 ± 0.87 | 339.5 ± 2.60 |

PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine. Trioleoylglycerol (TO) was emulsified with phospholipids or gum arabic, as described in Materials and Methods, and TO-hydrolyzing activity was measured in the presence and absence of ε-polylysine (2 μM). The results are expressed as mean ± SE of four experiments.

other emulsifiers (**Table 1**). In the presence of taurocholate, ε-polylysine strongly inhibited hydrolysis of TO-PC and TO-PE emulsions, but when the TO was emulsified with either PS or PA, the polymer stimulated TO hydrolysis slightly. When TO was emulsified with gum arabic instead of soybean PC, the TO-hydrolytic activity was reduced to about 20% (Table 1). This is likely due to its intrinsic surface activity as previously observed by others (23). However, ε-polylysine did not further inhibit TO hydrolysis. Also, ε-polylysine, up to 20 μM, did not affect the hydrolysis of the water-soluble substrate, methyl butyrate (data not shown). These results are consistent with the inhibitory effect of ε-polylysine being interfacial and show that inhibition is reduced or eliminated by the presence of anionic lipids or an anionic polymer (gum arabic) in the interface.

The hydrolysis of TO by the other abundant intestinal lipase, carboxylester lipase, was also inhibited by ε-polylysine, although the effective concentration required was twice as high (**Fig. 4**). BSA did not affect carboxylester lipase activity up to 1 mg/ml, but at 10 mg/ml it inhibited it completely (data not shown). The TO-hydrolyzing activity of lingual lipase was not inhibited by ε-polylysine but was stimulated slightly (Fig. 4). As lingual lipase activity was determined at pH 5.0, the effects of pH on the inhibition of pancreatic lipase activity were determined as a function of pH. ε-Polylysine (2 μM) inhibited TO-hydrolytic activity of pancreatic lipase at all pH values between 5.0 and 9.5 (data not shown), although it should be noted that loss of activity near pH 5 or pH 9 could be the result of the instability of pancreatic lipase (24). The concentration of ε-polylysine required to inhibit the cholesterol oleate-hydrolyzing activity of carboxylester lipase was about five times higher than that required to inhibit its TO-hydrolyzing activity (**Fig. 5**). Overall, these results are consistent with ε-polylysine inhibiting lipolysis at the interfacial level, as do other proteins (6), but with far greater potency. It does not, however, eliminate the possibility that the inhibitor could be interacting with lipase and carboxylester lipase in solution.

One way in which a water-soluble lipase can be inhibited by proteins at the interfacial level is to prevent its interaction with the substrate-containing interface (6). For

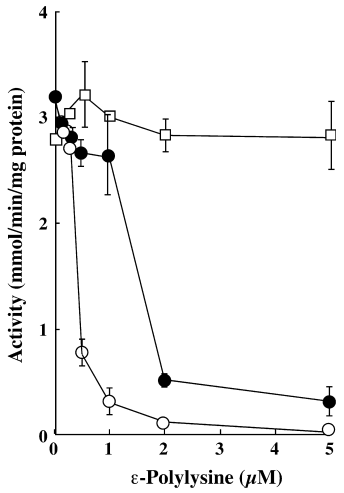


**Fig. 4.** Effect of increasing concentration of ε-polylysine on the rate of hydrolysis by digestive lipases of TO emulsified with PC. Experiments were performed in the presence of pancreatic lipase (open circle), carboxylester lipase (closed circle), and lingual lipase (square). Each point represents the mean ± SE of four separate experiments. For presentation on a common axis, the measured activities of carboxylester lipase and lingual lipase were multiplied by 20 and 10,000, respectively.

lipases, this adsorption step precedes the direct interaction of an individual substrate molecule with the active site of the enzyme. To determine if this was occurring in the present case, pancreatic lipase was incubated with TO-PC emulsion containing various amounts of ε-polylysine. After a 10 min incubation, the supernatant and lipid layer were separated by centrifugation and the pancreatic lipase protein levels in the supernatant and fat layer were estimated by Western blotting with an anti-pancreatic lipase antibody. **Figure 6A** shows representative immunoblots of pancreatic lipase in the supernatant and fat layer. ε-Polylysine reduced the amount of pancreatic lipase in the fat layer in a concentration-dependent manner and concomitantly increased that in the supernatant (Fig. 6B). Importantly, inhibition occurred over approximately the

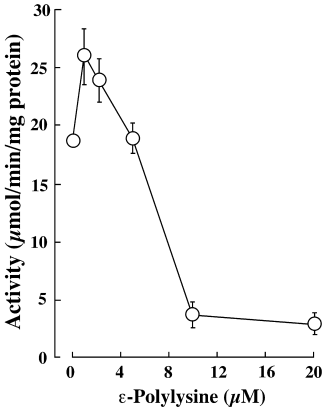


**Fig. 5.** Effects of increasing concentrations of ε-polylysine on the rate of hydrolysis by carboxylester lipase of cholesteryl oleate emulsified with soybean PC. Each point represents the mean ± SE of four separate experiments.

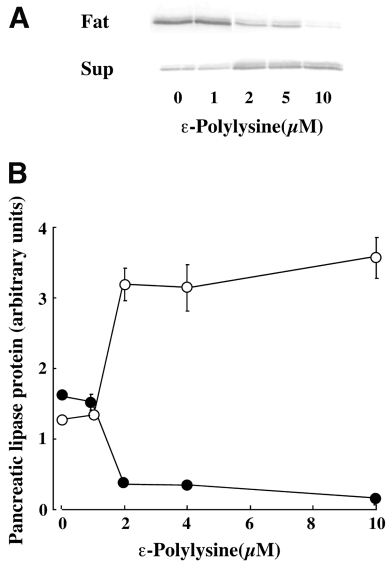


**Fig. 6.** Effect of ε-polylysine on pancreatic lipase distribution. Pancreatic lipase was incubated with a TO-PC emulsion containing various amounts of ε-polylysine. After a 10 min incubation, the supernatant and lipid layer were separated by centrifugation as described in Materials and Methods, and the proteins were separated by SDS-PAGE using gel containing 8% acrylamide. A: A representative immunoblot showing pancreatic lipase protein with various amounts of ε-polylysine. B: Pancreatic lipase immunoreactive protein in the supernatant (open circle) and fat layer (closed circle) in arbitrary units of density. Each data point is the mean ± SE of six separate experiments.

same range of inhibitor concentrations that blocked TO hydrolysis (Fig. 2). Thus, as for other, less potent inhibitory proteins, the mechanism of inhibition appears to involve a blocking of enzyme adsorption to the substrate-containing interface.

In the preceding experiments, the concentration of taurocholate was 0.265 mM, below its critical micellar concentration. This concentration was used so that only two phases were initially present in the assay system, the emulsion particles and the bulk aqueous phase, but the lipid-water interface was close to saturation with bile salt (25). For pancreatic lipase inhibition by other proteins, increasing bile salt to supramicellar concentration relieves inhibition (6), presumably by removing the inhibitor from the substrate-containing interface to micelles. To determine the sensitivity of pancreatic lipase inhibition to bile salt concentration, the hydrolysis of the PC-TO emulsion containing colipase was determined in the presence and absence of 1.0 μM ε-polylysine (**Fig. 7A**). The results show that in the absence of ε-polylysine, the enzyme retained most of its activity up to a taurocholate concentration of 8.0 mM, but that activity was largely inhibited at 10.0 mM detergent. With deoxycholate added in addition to the 0.265 mM present in the normal substrate emulsion, activity was maintained up to 1.0 mM before decreasing by about 50% at 2.0 mM. The taurocholate was present because emulsions prepared in its absence allow lipase activities only ~4% of those measured when it was included. The highest concentration with each bile salt is at or above its critical micellar concentration under similar conditions (26, 27). At all concentrations of taurocholate and deoxycholate tested, the presence of 1 μM ε-polylysine caused essentially complete inhibition of TO hydrolysis. In the presence of 7.5 mM taurocholate or 1.0 mM deoxycholate, the highest concentrations of bile salts that did not inhibit lipolysis in the absence of inhibitor, ε-polylysine concentrations as low as 0.4 μM caused nearly complete inhibition of lipolysis (Fig. 7B). The inability of high concentrations of bile salts either to reverse the inhibition of pancreatic lipase caused by ε-polylysine or to significantly alter its potency suggests some special property of this inhibitor protein compared with other proteins. Additionally, using substrate emulsions prepared in the absence of bile salt, inhibition by ε-polylysine was very weak; activity was decreased only 25% at 2.0 μM and 60% at 2.0 mM inhibitor.

### Monolayer studies

The results above suggest that ε-polylysine inhibits lipolysis by interrupting enzyme interface binding in a bile salt-dependent manner. However, not all emulsion surfaces

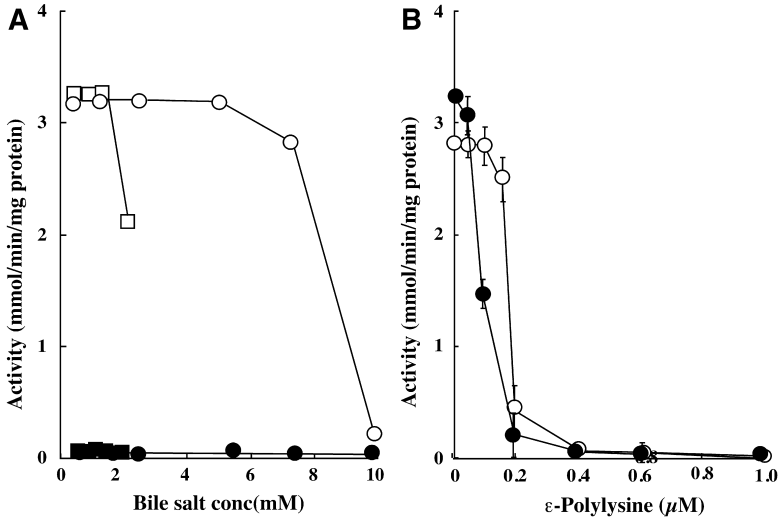


**Fig. 7.** Lack of reversal by supramicellar bile salts of the inhibition of pancreatic lipase by ε-polylysine. A: Activity with increasing concentration of deoxycholate (open square, closed square) or taurocholate (open circle, closed circle) in the presence (filled symbols) and absence (open symbols) of 1.0 μM ε-polylysine. B: Activity with increasing concentration of ε-polylysine in the presence of 1.0 mM deoxycholate (closed circle) or 7.5 mM taurocholate (open circle). Each point represents the mean ± SE of four separate experiments.

were equally affected, possibly because of differences in the ability of the zwitterionic emulsifiers to disperse the TO and other substrates. ε-Polylysine was a fairly weak inhibitor when bile salt was not present, but the low absolute rates complicate interpretation of the results. Therefore, we used monomolecular lipid films at the argon-buffer interface to help understand the potent inhibition of pancreatic lipase by ε-polylysine in the presence of bile salts. The advantage of this system is that the interfacial surface area is constant and independent of the lipids comprising the lipid monolayer or the additives present in the aqueous subphase supporting it.

The surface activity of [$^{14}$C]ε-polylysine in the absence of an insoluble lipid monolayer at the argon-buffer interface was first examined. With 100 μM taurocholate in the aqueous subphase, the initial surface pressure of the system, relative to buffer alone, was ~6 mN/m. Addition of ε-polylysine to the subphase produced a time-dependent increase in the surface pressure that equilibrated within <30 min (data not shown). For a series of such experiments, the dependence of the surface pressure increase on the concentration of ε-polylysine in the aqueous phase is shown in **Fig. 8A**. After equilibration of the surface pressure for each experiment, the interface was collected and the surface concentration of [$^{14}$C]ε-polylysine was determined (Fig. 8B, open circles). The equilibrium curves of the surface pressure change and the surface concentration of [$^{14}$C]ε-polylysine were similar, exhibiting saturation behavior. The binding indicated by both surface pressure and adsorption isotherms was analyzed using the Langmuir adsorption isotherm, assuming for Fig. 8A that the change in surface pressure is proportional to [$^{14}$C]ε-polylysine adsorbed (28). The analyses yielded similar dissociation constants of 35 nM and 43 nM, respectively. From the data in Fig. 8B, the maximum surface concentration of [$^{14}$C]ε-polylysine was calculated to be 12.7 pmol/cm$^2$. When taurocholate was omitted from the aqueous phase, however, the surface pressure did not increase following addition of [$^{14}$C]ε-polylysine (data not shown). Moreover, the binding of [$^{14}$C]ε-polylysine became essentially independent of its bulk concentration at a surface concentration less than one-fourth that obtained with taurocholate present (Fig. 8B, closed circles). Because taurocholate markedly enhances the surface activity and interfacial binding of [$^{14}$C]ε-polylysine, the data suggest formation of a surface-active complex between taurocholate and [$^{14}$C]ε-polylysine that partitions to the interface. Supporting this is the observation that in the concentration range of ε-polylysine over which most of the inhibition occurred, 0.2–0.4 μM (Fig. 2), the bile salt is in molar excess, i.e., the ratio of negative charge on the anionic detergent, taurocholate, to free amino groups on the polymer (~40 per molecule) ranged from six to three.

To determine whether the association of this complex with the interface would occur in the presence of the lipid species used to prepare the emulsions for experiments described above, additional binding measurements were carried out with 100 μM taurocholate present. [$^{14}$C]ε-polylysine was injected under preformed lipid monolayers of TO/1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC) (0.8:0.2; v/v) or SOPC alone. With a TO-SOPC mixed monolayer at an initial surface pressure of 16.3 ± 0.7 mN/m, [$^{14}$C]ε-polylysine adsorbed to a maximum surface concentration of 13.0 pmol/cm$^2$ (**Fig. 9A**; open circles) with a dissociation constant of 488 nM. At an initial surface pressure of 31.9 ± 0.2 mN/m, the dissociation constant was increased to 1,919 nM (Fig. 9B; open circles). However, the extrapolated maximum surface concentration of [$^{14}$C]ε-polylysine from the Langmuir binding analysis was essentially unchanged at 13.3 pmol/cm$^2$. Even with no TO present (Fig. 9C), [$^{14}$C]ε-polylysine adsorbed to an SOPC monolayer initially at 38.1 ± 0.2 mN/m to a maximum surface excess of 5.4 pmol/cm$^2$ with a dissociation constant of 720 nM. However, when the taurocholate concentration in the aqueous phase was decreased to 0.01 mM, the surface concentration of [$^{14}$C]ε-polylysine decreased to about one-third that observed with 0.1 mM taurocholate at TO-SOPC monolayers (Fig. 9A, B; open squares), and with no taurocholate present, little [$^{14}$C]ε-polylysine adsorbed to TO-SOPC or SOPC monolayers (Fig. 9A–C; filled circles). These data show clearly that binding of [$^{14}$C]ε-polylysine to lipid monolayers depends on the presence of taurocholate, supporting the notion of a surface-active taurocholate-polylysine complex being formed at the interface. Moreover, the binding was essentially saturated at ~2 μM [$^{14}$C]ε-polylysine, the concentration at which lipase binding and activity toward emulsions is completely blocked in the emulsion system.

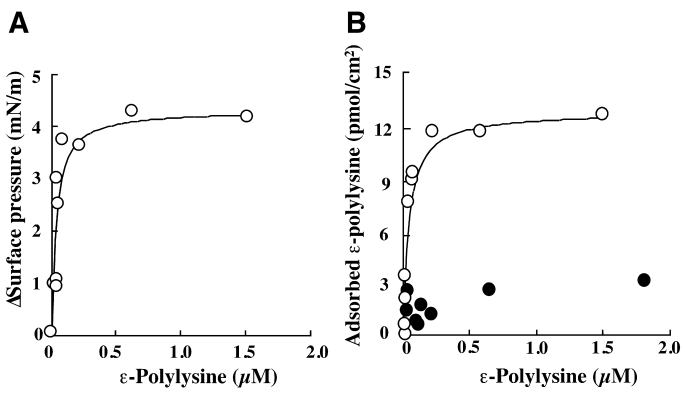


**Fig. 8.** [$^{14}$C]ε-polylysine interaction with the argon-buffer interface. Surface pressure (A) and ε-polylysine surface concentration (B) were measured 30 min after ε-polylysine was added to the aqueous phase with (open circle) or without (closed circle) taurocholate.

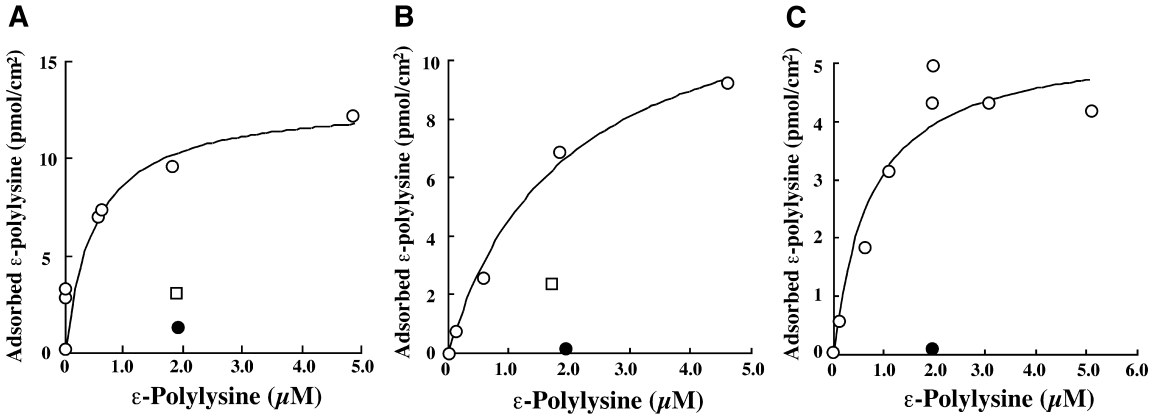


**Fig. 9.** Adsorption of [$^{14}$C]ε-polylysine to lipid monolayers. Lipid, TO/1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC) (0.8:0.2) (A, B), or SOPC (C) spread to a initial surface pressure of 16.3 ± 0.7 mN/m (A), 31.9 ± 0.2 mN/m (B), or 38.2 ± 0.2 mN/m (C). [$^{14}$C]ε-polylysine was injected to different final concentrations into the aqueous phase containing 0.1 (open circle), 0.01 (square), or 0 (closed circle) mM taurocholate. Thirty minutes after [$^{14}$C]ε-polylysine injection, lipid monolayer was collected and [$^{14}$C]ε-polylysine was determined as described in Materials and Methods.

In the lipase distribution experiments using substrate-containing emulsion particles (Fig. 6), ε-polylysine effectively blocked lipase adsorption. Binding of radiolabeled, catalytically inactive HPL, [$^{14}$C]HPL(S153G), was further investigated as a function of the monolayer surface pressure using TO-SOPC (0.8:0.2; v/v) monolayers on a taurocholate-containing subphase. At a bulk-phase concentration of 10.4 nM, its adsorption to the lipid monolayers spread to 10 mN/m, relative to the taurocholate subphase, was ~3 pmol/cm$^2$ (data not shown). This is a value about half that expected for formation of a complete [$^{14}$C]HPL(S153G) monolayer. However, increasing the pressure to 15 mN/m decreased binding by ~90% (data not shown). In the presence of 2.0 μM, ε-polylysine binding was reduced by >80% at 10 mN/m and even more at 15 mN/m (data not shown). Recalling that under comparable conditions, ε-polylysine increases surface pressure by ~4 mN/m (Fig. 8A), the data suggest that the ability of ε-polylysine to increase surface pressure could contribute to the inhibition of lipase adsorption at 10 mN/m. However, the presence of approximately equimolar colipase was able to partially overcome the inhibitory effects of both surface pressure and ε-polylysine at surface pressures of 10 mN/m and 15 mN/m, but not at higher pressures. The reasons for this are not clear, but the data suggest that increasing surface pressure alone does not explain the inhibitory effect of ε-polylysine on [$^{14}$C]HPL(S153G) adsorption.

## DISCUSSION

Dietary lipids are water-insoluble molecules and are emulsified by bile phospholipids. High concentrations of phospholipids are present in bile, and the major bile phospholipid is PC, comprising over 90% of the total. In the presence of PC and bile acids, the lipid surface at which the digestive enzyme can work is large. Dietary neutral lipids are presumed to be digested by three main enzymes: preduodenal, carboxylester, and pancreatic lipases (1). All of these are water-soluble enzymes and thus have access to the lipid only at the surface of the substrate lipid particle. Therefore, the lipase reaction occurs at the substrate surface (lipid-water interface) and is dependent upon surface adsorption of the enzyme. Other amphiphilic proteins are also adsorbed to (or penetrate) the interface and affect lipase activity (5, 6). Serum albumin has been shown to affect lipolysis in two different ways. At low concentrations, it protects the lipase from irreversible inactivation, and at high concentrations, it inhibits catalytic activity by blocking the substrate surface. Gargouri et al. (6, 29) reported that albumin and several other proteins, including melittin, β-lactoglobulin A, ovalbumin, and myoglobin, inhibited pancreatic lipase activity, and their inhibitory effects could be the result of lipase desorption from its substrate due to a change in interfacial quality. Bile salts were able to reverse this inhibition, presumably by removing these proteins from the substrate-containing interface.

In this study, we demonstrate that polylysine and ε-polylysine inhibit the hydrolysis of TO emulsified with PC with a potency that is two to three orders of magnitude greater than that of proteins used in earlier studies. The mechanism responsible for the inhibition seems, fundamentally, to be the same as that by which other amphiphilic proteins inhibit lipolysis, namely by nonspecifically blocking lipase adsorption to the interface (Fig. 6). Lipase inhibition by ε-polylysine depended on how substrate was presented to the lipases; it occurred with TO-PC and TO-PE emulsions, and not TO-PS, TO-PA, or TO-gum arabic stabilized emulsions. The distinction between the emulsifiers is that PC and PE are zwitterionic, whereas the others are anionic. Common to the emulsion systems in these assays was taurocholate, an anionic bile salt. With limiting taurocholate in emulsions, lipase inhibition occurred when the concentration of ε-polylysine positive charges exceeded by a few-fold the number of negative charges. Addition of a

large excess of taurocholate or deoxycholate only modestly increased the concentration of inhibitor needed to completely inhibit lipolysis. Indeed, emulsion experiments suggest that bile salt is required for potent inhibition to be observed. This is in sharp contrast to data for other inhibitory proteins, like albumin and β-lactoglobulin, that showed reversal of inhibition by supramicellar concentrations of bile salts (6).

Earlier work (6) showed that many proteins inhibit pancreatic lipase, irrespective of their charge at the assay pH. In the present case, the data suggest that charge is important, not with respect to the isoelectric point itself, but with respect to the number of positive charges per polylysine molecule (Fig. 3). The reason for this is suggested by the monolayer experiments with [$^{14}$C]ε-polylysine and taurocholate (Figs. 8, 9). These showed that the surface activity of the inhibitor, as measured both by surface pressure changes and by [$^{14}$C]ε-polylysine bound, increases in the presence of bile salt and saturates in the range at which the number of taurocholate negative charges exceeded the positive charges on [$^{14}$C]ε-polylysine. It should be noted that the radiolabeling procedure used does not change the net charge of the ε-polylysine, and the extent of labeling of amino groups was low. Combined, the results suggest that inhibition arises from the formation of a surface-active complex of ε-polylysine and taurocholate. Such a complex could contain up to 40 taurocholate molecules per ε-polylysine. The formation of nonabsorbable complexes between cationic polymers and bile salts is well known and provides the basis for cholesterol-lowering therapy, e.g., Questran and WelChol.

The inhibition requires bile salt and appears to be associated with formation of a surface-active ε-polylysine-bile salt complex that occupies the interface and thereby nonspecifically blocks lipase adsorption. Inhibition of the binding of [$^{14}$C]HPL(S153G) to monolayers, even at 15 mN/m, could be partially reversed by colipase, itself a surface-active protein. Thus, as observed previously (29), the ability of proteins or, in this case, an ε-polylysine-bile salt complex, to bind to the interface and increase surface pressure does provide a simple mechanism for the inhibition. Rather, earlier work has suggested that the rate of interactions of proteins with the interface, not measured in this study, is important (29). In this respect, inhibition by ε-polylysine may be like that of other proteins. Although the lack of inhibition of gastric lipase may seem to be a consequence of the absence of bile salt in that particular assay, ε-polylysine was only weakly inhibitory in its presence (data not shown). In this way, lingual lipase is like the lipase from *Rhizopus arrhizus* that is not inhibited by exogenous proteins under conditions in which pancreatic lipase is inhibited (6). Such resistance has been taken as evidence that inhibition of lipolysis by proteins does not occur by a simple masking of the substrate by the inhibitor as a consequence of its binding to the substrate-buffer interface (30).

The proposed role of an ε-polylysine-bile salt complex in the mechanism of inhibition is indirectly supported by the observation that the presence of anionic phospholipids (0.07 μmol) at a level higher than that of taurocholate (0.053 μmol) in the assay altered or prevented the inhibition. This could result in the formation of anionic phospholipid-polylysine complex that, for reasons not addressed, fails to inhibit lipase. One possibility is lateral phase separation of the complex on the substrate particle surface, rendering the rest of the surface vulnerable to lipase attack. However, this substitution could also have affected TO dispersion. Overall, the interfacial nature of the inhibition, although reasonable, is not unequivocally demonstrated in this study. This is because the possibility cannot be eliminated that the ε-polylysine-taurocholate complex that adsorbs to the interface stabilized by PC might also be present in solution, where it could bind lipase and prevent its binding to the interface. Nor do the present results unequivocally prove that the observed delay in triacylglycerol absorption in vivo (Fig. 1) results directly from the inhibition of lipolysis demonstrated in vitro (Fig. 2). An alternative possibility is that the polymer could sequester reaction products, thereby delaying their absorption. Resolving these issues will require additional studies, but the data, particularly the failure of high concentrations of bile salt to relieve the inhibition, are clearly consistent with ε-polylysine being able to act as an interfacial lipase inhibitor in the intestine.

Previously, we demonstrated that the basic protein, protamine, strongly inhibited hydrolysis of TO emulsified with PC (7). Protamine contains a large fraction of basic amino acid residues (arginine accounts for 40–70% of the total amino acids) and its isoelectric point is over pH 10. We proposed that protamine might bind to the PC coating on the TO, thereby inhibiting lipase activity, but did not explicitly consider the role of bile salt. The large number of positive charges of protamine and its isoelectric point >10 suggest that protamine may inhibit the lipase activity by the same mechanism as that proposed herein for polylysine and ε-polylysine. These results imply that any cationic polymer may act as a lipase inhibitor in the presence of bile salts in the digestive tract. ε-Polylysine may be superior to polylysine or protamine by virtue of its resistance to proteolysis. However, it remains to be seen if some long-term adaptation to such inhibitors may affect their ultimate utility.

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