Conjunctival Penetration of Insulin and Peptide Drugs in the Albino Rabbit

Eiji Hayakawa,[1,2] Du-Shieng Chien,[1,3] Kazuhiro Inagaki,[1,4] Akira Yamamoto,[1,5] Wei Wang,[1] and Vincent H. L. Lee[1]

Received August 28, 1991; accepted December 16, 1991

An *in vitro* model was used to evaluate the conjunctival penetration of three peptides, [D-ala^2]metenkephalinamide (YAGFM, MW 647), substance P (MW 1348), and insulin (MW 5778), in comparison with two nonpeptides, atenolol (MW 266) and timolol (MW 433). All three peptides were hydrolyzed to varying extents during penetration across the conjunctiva. The permeability coefficient for intact YAGFM and insulin was 4.5 ± 0.3 and 4.6 ± 0.7 μm sec^{-1}, respectively. These values were about two to five times lower than those for atenolol and timolol. No permeability coefficient could be calculated for substance P, since its transconjunctival flux never reached steady state. The conjunctival penetration of YAGFM and insulin was improved by about two and three times, respectively, with the addition of 1% Na glycocholate. Increasing the Na glycocholate concentration was more effective than changing the type of bile salt in improving the conjunctival penetration of insulin. The maximum factor of improvement was 12, as the Na glycocholate concentration was raised to 4%. The way in which Na deoxycholate, glycocholate, and taurocholate affected the conjunctival penetration of atenolol, timolol, and insulin suggests that these three bile salts improved mainly the transcellular penetration of the compounds studied.

KEY WORDS: beta blockers; conjunctival penetration; enkephalins; insulin; paracellular penetration; substance P; transcellular penetration.

INTRODUCTION

The conjunctiva is a thin mucous membrane covering the inside of the eyelid and the anterior sclera. It shares several features with other mucous membranes, such as those covering the nose and the intestine, namely, the presence of microvilli (1), an overlying mucus layer (2), and a rich blood supply in the lamina propia (3). But, unlike other mucous membranes, the conjunctiva is relatively accessible to experimental manipulation. This accessibility is advantageous, since it facilitates the investigation of formulation factors influencing the penetration of insulin and other peptide drugs across mucous membranes.

[1] University of Southern California, School of Pharmacy, Department of Pharmaceutical Sciences, 1985 Zonal Avenue, Los Angeles, California 90033.
[2] Present address: Kyowa Hakko Kogyo, Pharmaceutical Research Laboratories, Shizuoka, Japan.
[3] Present address: Allergan Pharmaceuticals, Irvine, California.
[4] Present address: Meijo University, Nagoya, Japan.
[5] Present address: Kyoto Pharmaceutical University, Department of Biopharmaceutics, Yamashina-ku, Kyoto, Japan 607.

All mucosal membranes are resistant to penetration by the majority of peptide and protein drugs (4). Consequently, penetration enhancers—typically low molecular solutes that alter either the permeability of mucosal membranes, the physicochemical properties of the penetrant, proteolytic activity, or the retention of the administered dose at the administration site (5)—are required for improving the bioavailability of these therapeutic entities. The relative contribution of the various mechanisms toward such improvements has never been well delineated. This is because the majority of the experiments was performed under conditions where all of the four possible mechanisms mentioned above were operative to varying extents.

Thus, the objectives of the present study were to investigate (a) how peptides of varying molecular size and susceptibility to proteolysis differed in their extents of penetration across the conjunctiva of the albino rabbit and (b) how the concentration and type of bile salts, a popular class of penetration enhancers (5), would alter the permeability of the conjunctiva to these drugs. The model peptides were [D-ala^2]metenkephalinamide (YAGFM), substance P, and insulin. Their molecular weights and sensitivities to proteolysis in conjunctival homogenates are listed in Table I. For comparison, two low molecular weight, nonpeptide drugs were also evaluated. They were the hydrophilic atenolol and the lipophilic timolol. The bile salts evaluated were Na deoxycholate (DC), Na glycocholate (GC), and Na taurocholate (TC), all at a concentration of 1%. In addition, the effect of Na glycocholate on conjunctival insulin penetration over the concentration range of 0.05 to 4% was also evaluated. Previous studies have revealed that YAGFM (6) and insulin (7) could penetrate the conjunctiva of the albino rabbit following topical instillation.

MATERIALS AND METHODS

Materials

Male, New Zealand albino rabbits, 1.8–2 kg, were purchased from Irish Farm Rabbitry (Los Angeles, CA). Atenolol, timolol maleate, [D-Ala^2]metenkephalinamide (YAGFM), substance P (SP) and its various hydrolytic fragments, reduced glutathione, Na deoxycholate, Na glycocholate, and Na taurocholate were purchased from Sigma Chemical Co. (St. Louis, MO). The SP hydrolytic fragments were the N-terminal fragments SP(1–2), SP(1–4), SP(1–7), and SP(1–9) and the C-terminal fragments SP(3–11), SP(5–11), SP(6–11), SP(7–11), SP(8–11), SP(9–11), methionine, and phenylalanine. Crystalline porcine Zn insulin was a gift from Lilly Research Laboratories (Indianapolis, IN). All reagents were of analytical grade and were used as received.

Conjunctival Penetration of Atenolol, Timolol, [D-Ala^2]Metenkephalinamide, Substance P, and Insulin

Rabbit conjunctivas were excised and mounted in modified Ussing chambers as described by Lee *et al.* (8). Fat and connective tissues from the serosal side of the palpebral conjunctiva were trimmed off before mounting in the chambers. Two and one-half milliliters of glutathione bicarbonate Ring-

0724-8741/92/0600-0769$06.50/0

Table I. Model Compounds Used in the Present Study, Their Molecular Weights (MW), and the Time ($t_{0.9}$) to Lose 10% of Their Initial Activities in Conjunctival Homogenates of the Albino Rabbit

Compound	MW	$t_{0.9}$ (min)[a]
Atenolol	266	Stable
Timolol	433	Stable
[D-Ala^2]Metenkephalinamide	647	53.9 ± 2.7
Substance P	1348	2.0 ± 0.14
Insulin	5778	43.1 ± 2.9

[a] From Ref. 17 for [D-Ala^2]metenkephalinamide, Ref. 18 for substance P, and Ref. 19 for insulin.

er's solution (GBR) (9), preadjusted to pH 7.4, were added to the serosal side. An equal volume of the same solution containing the penetrant, preadjusted to the desired pH and tonicity, was then added to the mucosal side. The initial concentration was 0.1 m*M* for insulin, 1 m*M* for YAGFM, 0.5 m*M* for substance P, and 3 m*M* for atenolol and timolol.

The contents of each chamber were mixed by bubbling a 95% O_2/5% CO_2 mixture at the rate of 3–4 bubbles/sec, and the temperature within each chamber was maintained at 37 ± 1°C by a circulating water bath. Under these conditions, the conjunctiva remained viable for as long as 240 min (10). At predetermined times up until 240 min (or 300 min in the case of substance P), 50-μl aliquots were sampled from the serosal side for assay of penetrant by reversed-phase HPLC and immediately replaced by an equal volume of GBR solution. The HPLC conditions are listed in Table II. The conditions for assaying insulin were also used to assay for the bile salts. The apparent permeability coefficient of each compound was calculated from the linear portion of a plot of the amount of penetrant accumulated vs time with proper corrections for the diffusional surface area and initial drug concentration. Moreover, for substance P, the area under the amount vs time curve for each substance P fragment was calculated by the trapezoidal rule. This parameter served, to a first approximation, as an index of the extent of formation of a given fragment from substance P.

Effect of Bile Salts on Conjunctival Drug Penetration

The effect of 1% Na glycocholate on the conjunctival penetration of 0.1 m*M* insulin, 1 m*M* YAGFM, 0.5 m*M* substance P, 3 m*M* atenolol, and 3 m*M* timolol was determined by including 1% Na glycocholate in the bathing solution containing the penetrant.

The influence of Na glycocholate concentration on the conjunctival penetration of 0.1 m*M* insulin, 3 m*M* atenolol, and 3 m*M* timolol was evaluated by including 0.05–4% Na glycocholate in the bathing solution containing the penetrant.

The role of the chemical nature of bile salts on conjunctival drug penetration was elucidated by including 1% Na deoxycholate, Na glycocholate, or Na taurocholate in the bathing solution containing 0.1 m*M* insulin, 3 m*M* atenolol, or 3 m*M* timolol.

RESULTS

Conjunctival Penetration of Insulin, YAGFM, Substance P, Atenolol, and Timolol

As shown in Fig. 1, insulin appeared on the serosal side only after 90 min. YAGFM, which had a permeability coefficient similar to that of insulin (Table III), appeared on the serosal side much sooner, at 30 min. The permeability coefficients of both peptides were, however, lower than those of atenolol and timolol (Table III). There was no evidence of insulin metabolism during conjunctival penetration. Although the deamidated metabolite of YAGFM contributed to about 30% of all the peptide recovered in the mucosal side at 240 min (data not shown), it did not appear on the serosal

Table II. Reversed-Phase HPLC Conditions for Insulin, [D-Ala^2]Metenkephalinamide (YAGFM), Substance P, Atenolol, and Timolol

Parameter	Insulin	YAGFM	Substance P	Atenolol	Timolol
Column	Vydac Protein & Peptide C18, 5 μm, 4.6 × 250 mm	Altex Ultrasphere ODS, 5 μm, 4.6 × 250 mm	Altex Ultrasphere ODS, 5 μm, 4.6 × 250 mm	Altex Ultrasphere ODS, 5 μm, 4.6 × 250 mm	Altex Ultrasphere ODS, 5 μm, 4.6 × 250 mm
Flow rate (ml/min)	1.1	1.0	1.0	1.0	1.0
Detection (nm)	UV 210	UV 214	UV 210	UV 281	UV 294
Mobile phase	A: 0.6% ethanolamine phosphate (pH 3.0)	A: 0.1 *M* $NaClO_4$ in 0.1% H_3PO_4 (pH 2.1)	A: 0.1 *M* $NaClO_4$ and 0.1% H_3PO_4 (pH 2.1)	A: 1% triethylamine HCl (pH 3.0)	A: 0.1% triethylamine HCl (pH 3.0)
	B: Acetonitrile	B: Acetonitrile	B: Acetonitrile	B: Acetonitrile	B: Methanol
Gradient	B. Concentration 17–40% in 37 min 40–17% in 42 min	B. Concentration 5–17% in 12 min 17–55% in 32 min 55% in 35 min 55–5% in 45 min	B. Concentration 10–12% in 7 min 12–20% in 16 min 20% in 20 min 20–35% in 30 min 35–37% in 35 min 37–54% in 42 min	B. Concentration 7% in 4 min 7–25% in 10 min 25% in 20 min 25–7% in 25 min	Isocratic B. 55%
Internal standard	Timolol maleate (0.2 mg/ml)	L-Tryptophan (0.1 mg/ml)	L-Tryptophan (0.1 mg/ml)	Timolol maleate (10 μg/ml)	Propranolol HCl (5 μg/ml)

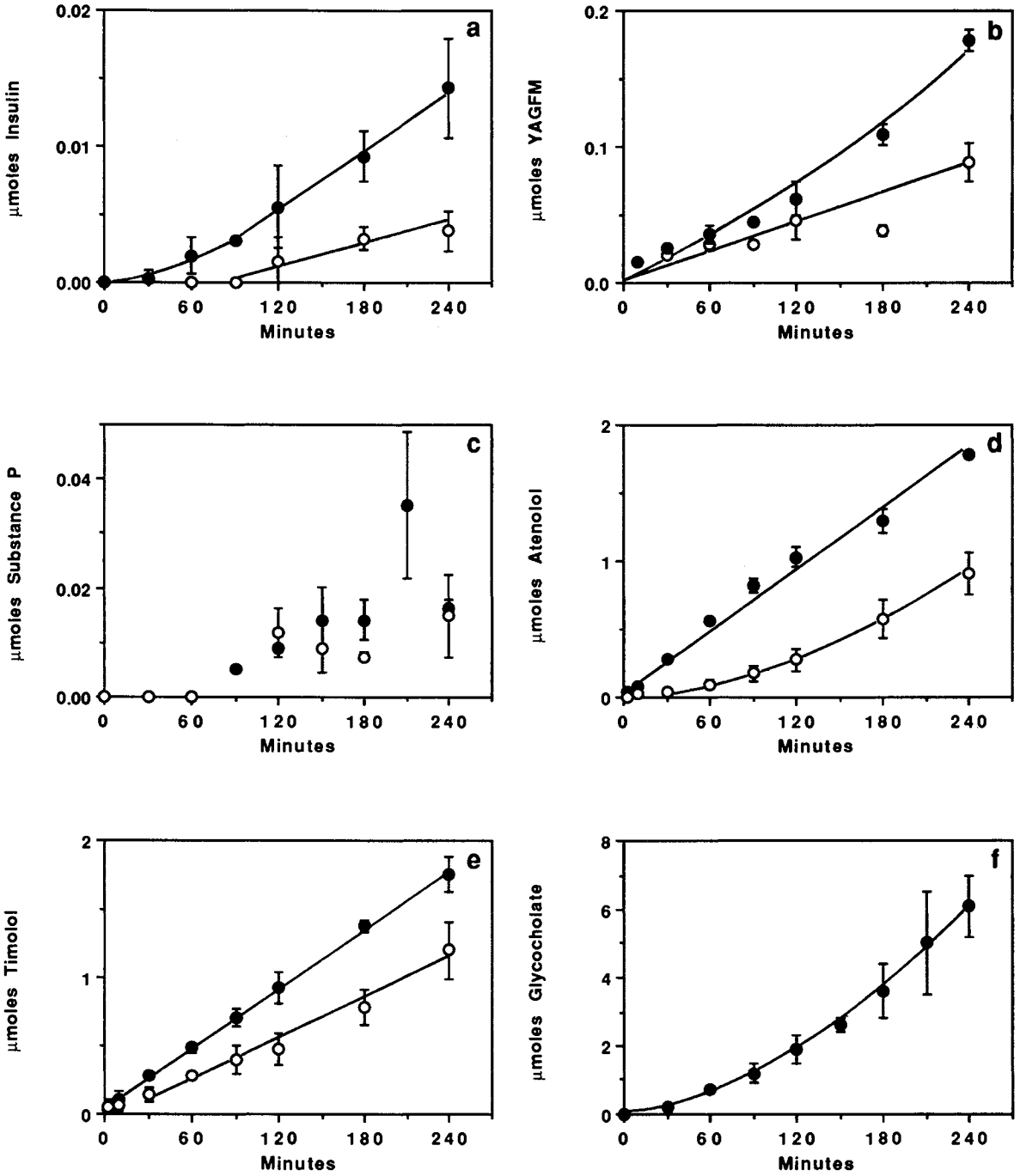

Fig. 1. Time course of conjunctival penetration of insulin (a), YAGFM (b), substance P (c), atenolol (d), and timolol (e) in the absence of 1% Na glycocholate (○) and in its presence (●) in the albino rabbit. The time course of conjunctival penetration of 1% Na glycocholate (f) is included for comparison. Only the intact peptide data are shown. The error bar represents standard error of the mean for n = 4–6. Where not shown, the error bar is smaller than the size of the symbol.

side. Metabolism was reduced to 8% in the presence of 1% Na glycocholate (data not shown).

Like insulin, substance P also penetrated the conjunctiva poorly. Unlike insulin, however, the principal reason for poor penetration of substance P was extensive metabolism both before and during penetration. At least 12 hydrolytic fragments—four N-terminal and eight C-terminal fragments—were recovered on the mucosal side, whereas 10 fragments were recovered on the serosal side (Fig. 2). The fragments that were recovered on both the mucosal and the serosal sides were the three N-terminal fragments, SP(1–2), SP(1–7), and SP(1–9), and the seven C-terminal fragments, SP(3–11), SP(5–11), SP(6–11), SP(8–11), SP(9–11), methionine, and phenylalanine. The fragments that were recovered on only the mucosal side were SP(1–4) and SP(7–11). The main fragments were SP(1–7), SP(1–9), SP(8–11), and phenylalanine on the mucosal side and SP(1–2), SP(9–11), methionine, and phenylalanine on the serosal side. Overall, the

Table III. Effect of 1% Na Glycocholate on the Conjunctival Permeability Coefficient (P_{app}) of Insulin, YAGFM, Substance P, Atenolol, and Timolol

Compound	P_{app} (10^5 cm sec^{-1})[a] Control	1% Na glycocholate	Enhancement factor[b]
Insulin	0.46 ± 0.07	1.38 ± 0.29	2.99
YAGFM	0.45 ± 0.03	0.85 ± 0.07	1.88
Substance P	—[c]	—	—
Atenolol	0.95 ± 0.31	4.39 ± 0.11	4.63
Timolol	2.21 ± 0.30	3.88 ± 0.20	1.76

[a] Mean ± SE; n = 3–5.
[b] Ratio of P_{app} in the presence of 1% Na glycocholate to P_{app} in its absence.
[c] Cannot be determined, since flux never reached steady state.

amount of hydrolytic fragments on the mucosal side was at least 10 times higher than that on the serosal side. The source of fragments was not determined for either the mucosal or the serosal side. The fragments on the mucosal side could be derived either from hydrolysis before penetration by proteases leached into the mucosal solution or from back-diffusion following hydrolysis by proteases in the conjunctiva during penetration. Those fragments recovered on the serosal side could be formed either before or during penetration across the conjunctiva.

Influence of 1% Na Glycocholate on Conjunctival Drug Penetration

Na glycocholate at 1% enhanced the conjunctival penetration of all solutes except substance P (Fig. 1 and Table III). The extent of enhancement was highest for atenolol, followed by insulin, YAGFM, and timolol, respectively.

In the presence of 1% Na glycocholate, the first-order rate constant of disappearance of substance P from the mucosal side was reduced 2.5 times, from 0.0091 to 0.0036 min^{-1} (data not shown). This finding was probably due to a reduction in the rate of hydrolysis, rather than to an increase in the rate of penetration across the conjunctiva. Based on the area under the amount vs time curve, there was a 440% reduction in the extent of formation of N-terminal fragments and a 20% reduction in the formation of C-terminal fragments (Fig. 2). There was, however, an increase in the formation of SP(3–11), SP(5–11), and SP(6–11). As was the case without Na glycocholate, there were approximately 10 times more hydrolytic fragments on the mucosal than on the serosal side. The relative proportions of various SP hydrolytic fragments on the serosal side were altered by 1% Na glycocholate (Fig. 2), reflecting its effect on both metabolism of substance P and penetration of its hydrolytic fragments.

Influence of Na Glycocholate Concentrations on Conjunctival Drug Penetration

The permeability coefficient of insulin increased monotonically with Na glycocholate concentration (Fig. 3a). This trend was unlike that of the permeability coefficient of Na glycocholate, which reached a plateau beyond 3% (Fig. 3b). Concurrent with the increase in P_{app}, there was a reduction in lag time (Fig. 4).

In the case of atenolol and timolol, the P_{app} vs Na glycocholate concentration plot is best described by a sigmoidal relationship with an inflection point at between 0.8 and 1% Na glycocholate and a plateau in P_{app} at 2% Na glycocholate (Figs. 3c and d).

Relative Effectiveness of Bile Salts on Conjunctival Drug Penetration

The relative effectiveness of 1% Na deoxycholate, Na glycocholate, and Na taurocholate in enhancing conjunctival drug penetration was drug dependent. Na deoxycholate was somewhat more effective than Na glycocholate and Na taurocholate in enhancing the conjunctival penetration of insulin (Fig. 5a). This pattern was consistent with the rank order of the P_{app}'s of the bile salts themselves (Fig. 5b). For timolol, the rank order of effectiveness was Na glycocholate = Na taurocholate > Na deoxycholate > control (Fig. 5d). More prominent differences were seen in atenolol; the rank

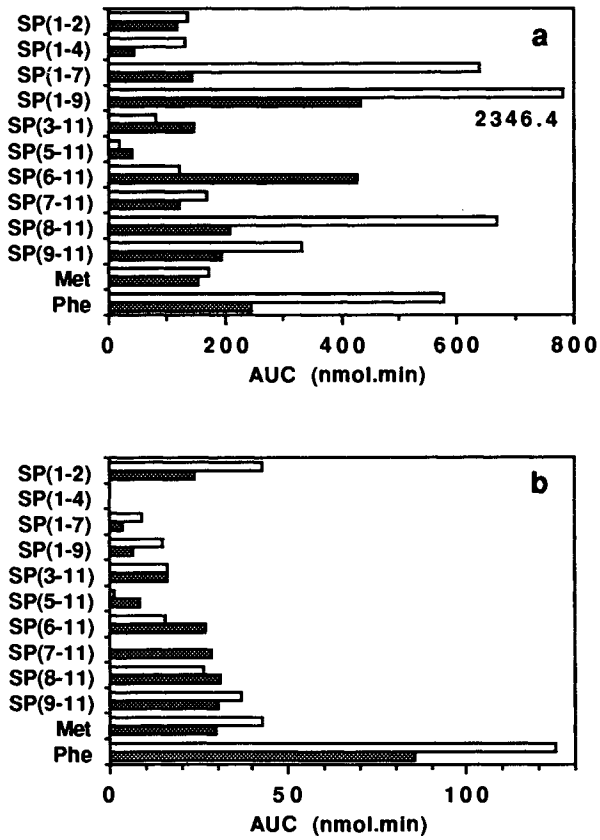

Fig. 2. Area under the amount vs time curve from 0 to 300 min (AUC) of substance P fragments on the mucosal side (a) and on the serosal side (b) of the isolated albino rabbit conjunctiva both in the absence of (open bars) and in the presence of (hatched bars) 1% Na glycocholate. The AUC for substance P was 132,298 and 3118 nmol · min on the mucosal and serosal sides, respectively, in the absence of 1% Na glycocholate and 227,957 and 3996 nmol · min on the mucosal and serosal sides, respectively, in the presence of 1% Na glycocholate. The value shown for SP(1–9) on the mucosal side in the absence of 1% Na glycocholate is one-third the actual value, in order for all the bars in that plot to be visible.

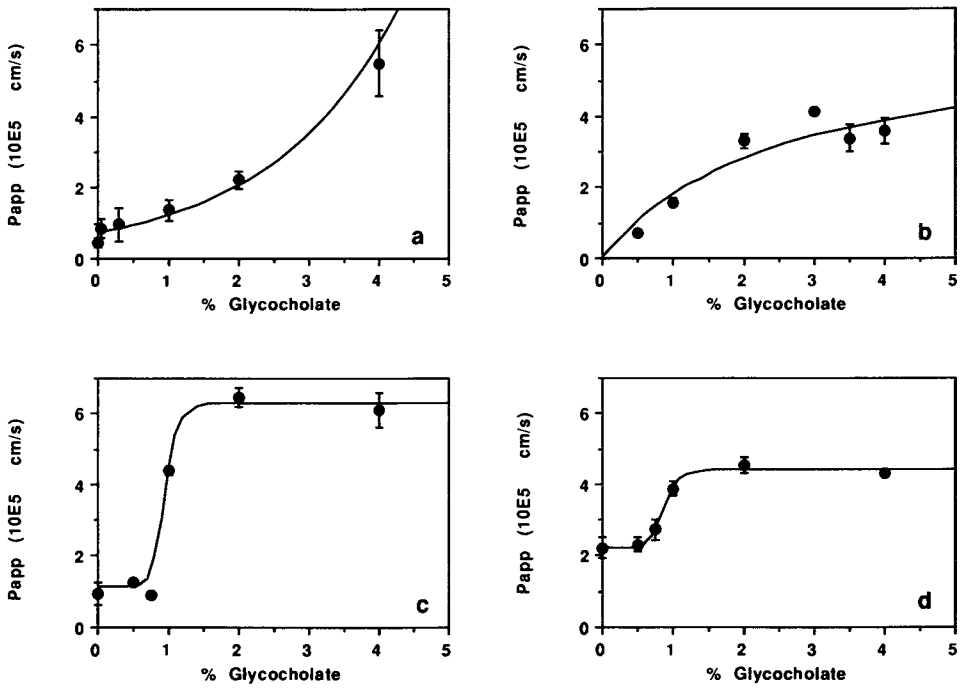

Fig. 3. Influence of Na glycocholate concentration on the conjunctival permeability coefficients (P_{app}) of insulin (a), Na glycocholate (b), atenolol (c), and timolol (d) in the albino rabbit. The error bar represents the standard error of the mean for n = 4–6. Where not shown, the error bar is smaller than the symbol.

order of effectiveness was Na glycocholate > Na deoxycholate ≫ Na taurocholate = control (Fig. 5c). The above rank orders were found to be statistically different for each drug based on one-way ANOVA at $P < 0.05$.

DISCUSSION

As expected, the three peptides, YAGFM, substance P, and insulin, penetrated the conjunctiva to a lesser extent than the two nonpeptides, atenolol and timolol (Fig. 1 and Table III). Conjunctival peptide penetration was not, however, determined by molecular size alone. This is indicated by the similar permeability coefficients of insulin and YAGFM despite marked differences in molecular size (Table III). Unlike YAGFM and insulin, which were metabolized only to a small extent or not at all during conjunctival penetration, substance P was extensively metabolized. Its conjunctival penetration was not improved by Na glycocholate (Fig. 1c), indicating that the metabolic barrier was a formidable barrier to its conjunctival penetration.

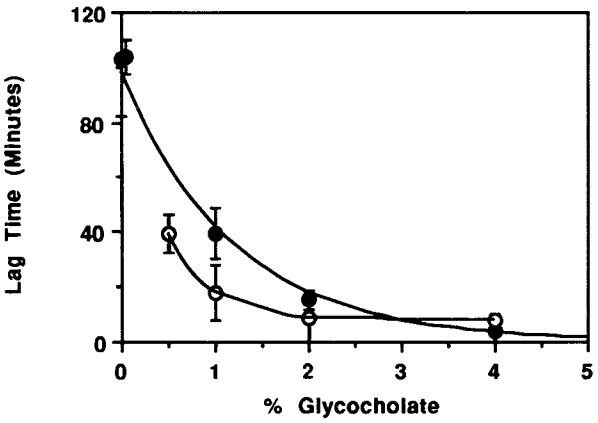

Fig. 4. Influence of Na glycocholate concentration on the lag time of penetration of insulin (●) and Na glycocholate (○) across the conjunctiva of the albino rabbit. The error bar represents the standard error of the mean for n = 4–6. Where not shown, the error bar is smaller than the symbol.

The conjunctival penetration of insulin was improved three times by 1% Na glycocholate, compared with 1.8 times for timolol and 4.6 times for atenolol. Comparing Figs. 3 and 5 reveals that increasing the Na glycocholate concentration to 4% is more effective than changing it to another type of bile salt in improving the conjunctival penetration of insulin as well as the conjunctival penetration of atenolol. The threshold effective Na glycocholate concentration for enhancement of atenolol and timolol penetration is about four times higher than the critical micellar concentration reported for this bile salt, 0.2% (11–13) (Figs. 3c and d). This threshold concentration is, however, somewhat lower for enhancement of insulin penetration (Fig. 3a). It is surprising that Na deoxycholate, a very aggressive penetration enhancer (14), was only marginally more effective than the less aggressive Na glycocholate in enhancing insulin penetration (Fig. 5a).

An obvious mechanism by which Na glycocholate improved the conjunctival penetration of insulin, YAGFM, atenolol, and timolol was weakening of the barrier function of either the apical membrane of the epithelial cells or the junctions between them. Another possible way of improving penetration is loosening the mucus layer overlying the epithelial cells (5). Since there is no evidence for insulin degradation during penetration, it is unlikely that protection of insulin from proteolysis by Na glycocholate reported previously for nasal insulin absorption (15) is a mechanism. How-

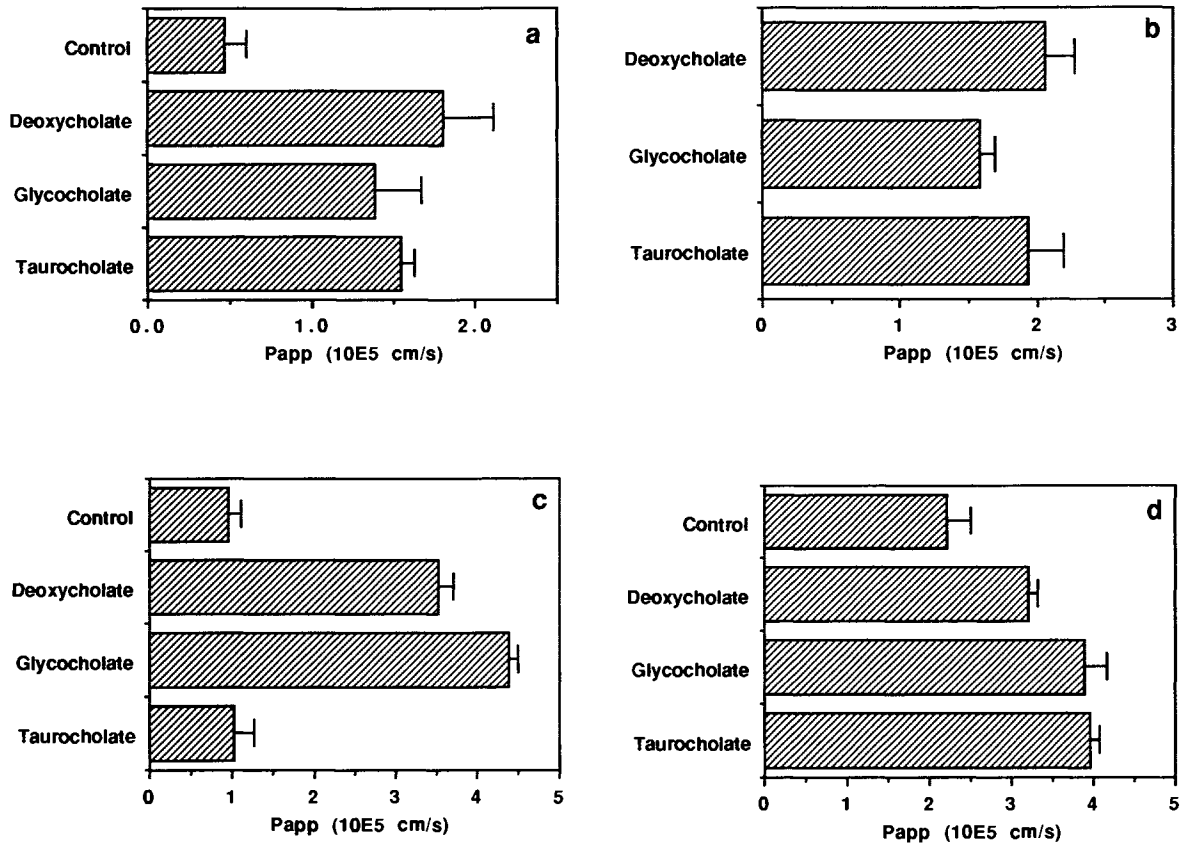

Fig. 5. Influence of type of bile salt on the conjunctival permeability coefficients (P_{app}) of insulin (a), bile salts (b), atenolol (c), and timolol (d) in the albino rabbit. The error bar represents the standard error of the mean for n = 4–6. Where not shown, the error bar is smaller than the symbol.

ever, the possible mechanism of a shift in the insulin monomer–multimer equilibrium toward the monomer cannot be ruled out. Such a shift is indicated by the increase in the fraction of insulin monomers at 0.1 m*M* capable of passing through a dialysis membrane with a MW cutoff of 10,000 Dalton's (Molecut UFP1 TGC, Millipore, Yonezawa, Japan) under ultrafiltration. This fraction was 0.46% at 0.1% Na glycocholate, 0.65% at 0.5% Na glycocholate, 2.4% at 1% Na glycocholate, 18.8% at 2% Na glycocholate, and 38.9% at 4% Na glycocholate.

The cellular locus of action is probably different for the three bile salts studied. On the expectation that atenolol penetrates the conjunctiva primarily via the paracellular pathway and that timolol penetrates the conjunctiva primarily via the transcellular pathway—as is the case in brain vascular endothelium (16)—we speculate that the lack of an effect by Na taurocholate on conjunctival atenolol penetration is suggestive of a primary, albeit mild, effect on the membrane. That Na glycocholate and Na deoxycholate improved the conjunctival penetration of both atenolol and timolol suggests a dual action on the membrane and the tight junction. The higher maximum P_{app} attained by atenolol (a paracellular penetrant), compared with timolol (a transcellular penetrant), suggests that Na glycocholate is more active at the paracellular than at the transcellular pathway (Figs. 3c and d). Although direct confirmation is required, the similar magnitude of increase in conjunctival insulin penetration caused by all three bile salts (Fig. 5a), in spite of marked differences in their effect on epithelial integrity, suggests improved transcellular rather than paracellular transport of insulin by the bile salts. An alternative interpretation of this finding is that either of the two mechanisms discussed above—solubilization of insulin or, to a limited extent, disruption of the mucus layer overlying the conjunctival mucosa—is the overriding factor in the improvement of conjunctival insulin penetration by the addition of bile salts. The fraction of insulin monomers at 0.1 m*M* capable of passing through a dialysis membrane with a MW cutoff of 10,000 Dalton's (Molecut UFP1 TGC, Millipore, Yonezawa, Japan) under ultrafiltration was 5.8% for 1% Na deoxycholate, 2.4% for 1% Na glycocholate, and 1.0% for 1% Na taurocholate.

In conclusion, the conjunctiva of the albino rabbit permits the passage of peptides such as YAGFM, substance P, and insulin to varying extents. Compared with atenolol and timolol, penetration of these peptides is low. But it can be improved by the addition of bile salts such as Na deoxycholate, Na glycocholate, and Na taurocholate at concentrations above their critical micellar concentrations. The mechanism of enhancement is probably due to a combination of solubilization of insulin and a lowering of the barrier function of the conjunctiva at the level of the plasma membranes. On this basis, the conjunctiva responds to the penetration enhancement effect of bile salts in a manner similar to that of other mucosal membranes.

ACKNOWLEDGMENTS

This work was supported by Grants EY3670 and EY7389 from the National Institutes of Health, Bethesda, Maryland, and by the Gavin S. Herbert Professorship.

REFERENCES

1. R. R. Pfister. The normal surface of conjunctiva epithelium. A scanning electron microscopic study. *Invest. Ophthalmol.* 14:267–279 (1975).
2. B. A. Nichols, M. L. Chiappino, and C. R. Dawson. Demonstration of the mucous layer of the tear film by electron microscopy. *Invest. Ophthalmol. Vis. Sci.* 26:464–473 (1985).
3. P. C. Y. Chen, S. W. Kovalcheck, and B. W. Zweifach. Analysis of microvascular network in bulbar conjunctiva by image processing. *Int. J. Microcirc. Clin. Exp.* 6:245–255 (1987).
4. V. H. L. Lee and A. Yamamoto. Penetration and enzymatic barriers to peptide and protein absorption. *Adv. Drug Deliv. Rev.* 4:171–207 (1990).
5. V. H. L. Lee, A. Yamamoto, and U. B. Kompella. Mucosal penetration enhancers for facilitation of peptide and protein drug absorption. *CRC Crit. Rev. Drug Carriers Syst.* 8:91–192 (1991).
6. R. E. Stratford, Jr., L. W. Carson, S. Dodda Kashi, and V. H. L. Lee. Systemic absorption of ocularly administered enkephalinamide and inulin in the albino rabbit: Extent, pathways, and vehicle effects. *J. Pharm. Sci.* 77:838–842 (1988).
7. A. Yamamoto, A. M. Luo, S. Dodda-Kashi, and V. H. L. Lee. The ocular route for systemic insulin delivery in the albino rabbit. *J. Pharmacol. Exp. Ther.* 249:249–255 (1989).
8. V. H. L. Lee, D. S. Chien, and H. Sasaki. Ocular ketone reductase distribution and its role in the metabolism of ocularly applied levobunolol in the pigmented rabbit. *J. Pharmacol. Exp. Ther.* 246:871–878 (1988).
9. H. F. Edelhauser, J. R. Hoffert, and P. O. Fromm. In vitro ion and water movement in corneas of rainbow trout. *Invest. Ophthalmol.* 4:290–296 (1965).
10. D.-S. Chien, H. Sasaki, H. Bundgaard, A. Buur, and V. H. L. Lee. Role of enzymatic lability in the corneal and conjunctival penetration of timolol ester prodrugs in the pigmented rabbit. *Pharm. Res.* 8:728–733 (1991).
11. G. W. Gullikson, W. S. Cline, V. Lorenzsonn, L. Benz, W. A. Olsen, and P. Bass. Effects of anionic surfactants on hamster small intestinal membrane structure and function: Relationship to surface activity. *Gastroenterology* 73:501–511 (1977).
12. T. Murakami, Y. Sasaki, R. Yamajo, and N. Yata. Effect of bile salts on the rectal absorption of sodium ampicillin in rats. *Chem. Pharm. Bull.* 32:1948–1955 (1984).
13. K. Miyajima, M. Yokoi, H. Komatsu, and M. Nakagaki. Interaction of β-cyclodextrin with bile salts in aqueous solutions. *Chem. Pharm. Bull.* 34:1395–1398 (1986).
14. M. A. Wheatley, J. Dent, E. B. Wheeldon, and P. L. Smith. Nasal drug delivery: An in vitro characterization of transepithelial electrical properties and fluxes in the presence or absence of enhancers. *J. Control. Rel.* 8:167–177 (1988).
15. S. Hirai, T. Yashiki, and H. Mima. Mechanisms for the enhancement of the nasal absorption of insulin by surfactants. *Int. J. Pharm.* 9:173–184 (1981).
16. J. B. Van Bree, A. G. De Boer, M. Danhof, L. A. Ginsel, and D. D. Breimer. Characterization of an "in vitro" blood-brain barrier: Effects of molecular size and lipophilicity on cerebrovascular endothelial transport rate of drugs. *J. Pharmacol. Exp. Ther.* 247:1233–1239 (1988).
17. S. Dodda-Kashi and V. H. L. Lee. Hydrolysis of enkephalins in homogenates of anterior segment tissues of the albino rabbit eye. *Invest. Ophthalmol. Vis. Sci.* 27:1300–1303 (1986).
18. V. H. L. Lee, S. Dodda Kashi, R. M. Patel, E. Hayakawa, and K. Inagaki. Mucosal peptide and protein delivery: Proteolytic activities in mucosal homogenates. *Proc. Int. Symp. Control Rel. Bioact. Mater.* 14:23–24 (1987).
19. A. Yamamoto, E. Hayakawa, and V. H. L. Lee. Insulin and proinsulin proteolysis in mucosal homogenates of the albino rabbit: Implications in peptide delivery from nonoral routes. *Life Sci.* 47:2465–2474 (1990).