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Toxicological and absorption enhancing effects of glycofurol 75 and sodium glycocholate in monolayers of human intestinal epithelial (Caco-2) cells

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Summary

The effects of glycofurol 75 (GF) and sodium glycocholate (GC) on the toxicity and permeability of the human intestinal cell line Caco-2 were studied. The intracellular dehydrogenase activity of the cells was used as a measure of the toxicity. Concentrations of GC from approx. 10 mM inhibited the intracellular dehydrogenase activity and above 40 mM the activity was less than 10% of the initial level. The concentrations resulting in 50% inhibition (IC_{50}) were 24.2 mM (1.2%) and 380 mM (6.8%) for GC and GF respectively. GF concentrations of less than 100 mM did not affect the activity. The effects of GF and GC on the absorption of the hydrophilic marker molecule [^{14}C]mannitol were studied at concentration levels corresponding to no (23.7 mM GF and 5.1 mM GC), about 25% (117 mM GF and 17.2 mM GC) and 50% (380 mM GF and 24.2 mM GC) inhibition in dehydrogenase activity. The apparent permeability coefficient for mannitol in control monolayers was 5.7×10^{-8} cm/s. 5.1 mM GC did not enhance the permeability, whereas 17.2 and 24.2 mM enhanced it significantly ($p < 0.001$). GF (380 mM) did not enhance the permeability. The apparent permeability coefficient of insulin in control monolayers was $\leqq 9.8 \times 10^{-8}$ cm/s, but varied considerably. 24.2 mM GC enhanced the P_{app} significantly ($p < 0.001$), whereas GF (380 mM) did not affect the absorption of insulin. The Caco-2 cells were studied by transmission electron microscopy after exposure to 380 mM GF for 1 h. Cells exposed to a mannitol solution of the same osmolality as 380 mM GF (about 700 mosm) and untreated monolayers of Caco-2 cells served as controls. GF caused morphological alteration of the epithelial cells resulting in a distorted appearance with disordered microvilli, disorganized terminal web and intracellular vacuols. This effect is problaby due to the high osmolality as the cells exposed to mannitol displayed the same distorted appearance. However, no effect could be observed on the integrity of the apical cell membrane and the tight junctions.

Introduction

Recent developments in biotechnology and therapeutics have increased the number of peptide and protein drugs. A substantial disadvantage of these drugs is related to their chemical and physical properties such as molecular size, hydrophilicity, susceptability to proteolytic breakdown, first pass metabolism and denaturation, which make them unsuitable for delivery by the oral route. It has been possible to find alternative administration routes for smaller peptides such as vasopressin, oxytocin and LHRH, which are now

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available as nasal preparations. However, for larger peptides and proteins, parenteral administration (s.c., i.m. and i.v.) is the only way to obtain a reasonable systemic activity. In many cases, especially in long term treatments, it is undesirable to use parenteral administration only. To fully utilize the larger peptide and protein drugs in the future, alternative routes and delivery systems must be found.

The nasal route is characterized by features important for systemic delivery such as a highly vascularized mucosal bed (Mygind, 1985) and the avoidance of first pass metabolism. Additionally, the drug is readily administered, dosage is reproducible and rapid absorption is possible. Unfortunately, good absorption can only be expected for compounds with a molecular weight of less than 1000 (McMartin et al., 1987).

One way to increase the nasal bioavailability of larger peptide and protein drugs has been by coadministration of absorption enhancers. A drawback of most absorption enhancers is their toxicological effect, e.g., cell erosion, excessive mucus discharge, membrane protein removal and mucociliotoxicity (Hirai et al., 1981b; Duchateau et al., 1986; Hersey and Jackson, 1987; Gizurarson et al., 1990; Hermens et al., 1990b). Many different compounds have been studied in order to find an acceptable balance between absorption enhancing and toxicological effects (Ennis et al., 1990; Chandler et al., 1991). Among the absorption enhancers studied for nasal administration are bile acids (Hirai et al., 1981a; Duchateau et al., 1986; Donovan et al., 1990), surfactants, e.g., laureth-9 (Donovan et al., 1990), cyclodextrins (Hermens et al., 1990a; Merkus et al., 1991), phospholipids (Hansen et al., 1988; Illum et al., 1989) and fatty acids (Mishima et al., 1987). Sodium glycocholate (GC) is an example of an absorption enhancer claimed to have a relatively low local toxicity (Hirai et al., 1981b; Gizurarson et al., 1990; Hermens et al., 1990b) and good absorption enhancing properties (Hirai et al., 1981a; Chan et al., 1988; Pontiroli et al., 1989).

Glycofurol 75 (GF) is a substance which is usually employed as a cosolvent in parenteral solutions of lipophilic drugs (Spiegel et al., 1956). Bechgaard et al. (1991) have recently shown that GF is also usable as an adjuvant (absorption enhancer) in a nasal insulin preparation. In vivo addition of 5% GF substantially enhanced the absorption of insulin in rabbits, but how GF influences the absorption is not known. Further, knowledge concerning the local nasal toxicity of GF is limited (Bechgaard et al., 1991).

Various epithelial cell cultures have recently been introduced as models of drug absorption. Of these, the Caco-2 cell line has been widely utilized to study drug transport (Artursson, 1990; Artursson and Magnusson, 1990; Lundin and Artursson, 1990; Wilson et al., 1990) as well as absorption enhancement (Anderberg et al., 1992, 1993; Iso-Aho and Artursson, 1992; Anderberg and Artursson, 1993).

The objective of this study has been to characterize the two absorption enhancers, GF and GC, concerning their toxicological and absorption enhancing properties in Caco-2 cell monolayers.

Materials and methods

Chemicals

Zinc-free human insulin was kindly provided by Novo Nordisk (Bagsværd, Denmark). [^{14}C]-Mannitol (Mol. Wt 182, specific radioactivity 275 mCi/g) was obtained from New England Nuclear (Boston, MA, U.S.A.) through Du Pont Scandinavia A.B. (Kista, Sweden). Synthetic sodium glycocholate (GC; approx. 99% purity) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT; 98% purity) were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). Glycofurol 75 (GF) was obtained from Roche (Basle, Switzerland).

Cells

Caco-2 cells originating from a human colorectal carcinoma were obtained from the American Tissue Culture Collection (Rockville, MD, U.S.A.). The cells were cultivated on polycarbonate filters (Costar Transwell cell culture inserts; mean pore diameter 0.45 μm) as described by Artursson (1990). Cells of passage 94–102 were used. All tissue culture media were obtained from

Gibco through Laboratorie Design AB (Lingingö, Sweden).

Osmolality

The osmolalities were measured in a 5500 Vapor Pressure Osmometer (Wescor Inc., Logan, UT, U.S.A.).

Intracellular enzyme activity

The effects of GF and GC on the intracellular dehydrogenase activity were determined by the MTT method (Mosmann, 1983; Tada et al., 1986). MTT is a tetrazolium salt that is cleaved in living cells by mitochondrial dehydrogenases, to give dark blue formazan crystals. Cells were seeded in 96-well tissue culture plates (Flow Laboratories, Herts, U.K.) and incubated for 24 h. A cell concentration of 50000 cells/well was used in all experiments since Anderberg et al. (1992) have shown a linear correlation between cell number and dehydrogenase activity in the range of 8000–62 500 cells/well. The cells were incubated for 10 min with different concentrations of GF and GC, whereafter MTT solution (5 mg/ml in phosphate-buffered saline) was added and the plates were incubated for another 90 min. A solution consisting of 10% sodium dodecyl sulfate and 0.01 M HCl in isobutanol was added to solubilize the cell membranes and dissolve the formazan crystals. The colour was measured at $\lambda = 590$ nm in a multiwell scanning spectrophotometer (Multiscan MCC/340 Labsystems Oy, Helsinki, Finland). The concentrations of GF and GC producing 50% inhibition of the dehydrogenase activity (IC_{50}) were determined.

Absorption and recovery study

The absorption of [^{14}C]mannitol and insulin (initial concentration 2×10^{-5} M and 4 IU/ml, respectively) across monolayers of Caco-2 cells was studied in air at 37°C and 95% relative humidity in Hanks' Balanced Salt Solution (HBSS). The drug solutions were added to the apical side and samples were taken at regular time intervals from the basolateral side. The monolayers were agitated on a microscope slide mixer (Relax 3, Kebo Lab, Sweden).

The effects of GF and GC on the absorption rate of mannitol were studied at three concentrations for each: 23.7, 117 and 380 mM GF; and 5.1, 17.2 and 24.2 mM GC, representing zero, about 25% and 50% inhibition at the enzyme dehydrogenase activity, respectively. The effects of GF and GC on the absorption rate of insulin were tested at the highest concentrations only.

The recovery of the monolayers were studied by determination of the absorption rate of mannitol (initial concentration 1×10^{-5} M) before, during and after addition of 10.25 mM GC (= the highest examined concentration of GC which did not affect the dehydrogenase activity). Initially, the monolayers were exposed to HBSS containing [^{14}C]mannitol for 1 h. The apical medium was then changed to HBSS containing 10.25 mM GC and [^{14}C]mannitol and incubated for another 20 min. Afterwards both the apical and basolateral media were replaced with Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum and 1% non-essential amino acids and incubated for 1 h. The more nutritious medium was required in order to achieve rapid recovery of the monolayers. Finally, the apical solution was replaced with a solution of DMEM containing [^{14}C]mannitol and the monolayers were incubated for another 1 h (Anderberg et al., 1992). The media contained 0.01% penicillin/streptomycin (PEST).

Similar experiments, but where the addition of GC and 0.01% PEST was excluded, were used as controls.

Analysis

10 ml of scintillant were added to samples of mannitol (500 μl) and counted in a liquid scintillation spectrometer (Tricarb 1900 CA, Packard Instruments).

Insulin was analysed by radioimmunoassay (Pharmacia Insulin RIA 100, Kabi-Pharmacia, Uppsala, Sweden).

Calculation

The apparent permeability coefficient (P_{app}) was calculated using the following equation:

$$P_{app} = \frac{dQ}{dt}\frac{1}{CA} \text{ (cm/s)}$$

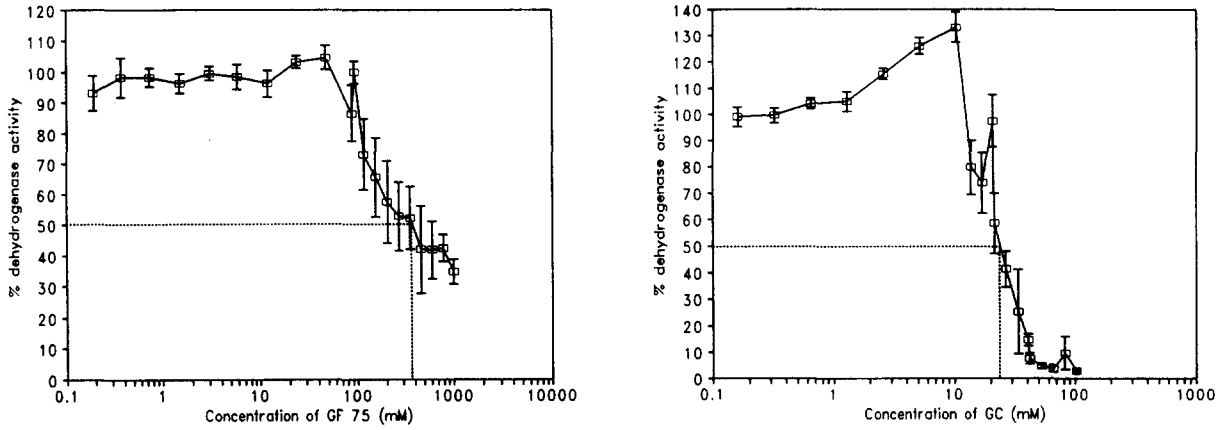

Fig. 1. Effect of (a) glycofurol 75 (GF) and (b) sodium glycocholate (GC) on intracellular dehydrogenase activities (mean ± S.D., $n = 8$). IC_{50} values are indicated by dashed lines.

where dQ/dt is the permeability rate of mannitol or insulin (steady state flux mol/s or μU/s, respectively), C denotes the initial apical concentration of mannitol (mol/ml) or insulin (IU/ml) and A is the surface area (cm^2) of the membrane.

Transmission electron microscopy (TEM)

The monolayers of Caco-2 cells were incubated with HBSS containing 380 mM GF (osmolality ≈ 700 mosm) for 1 h, and then fixed with formalin. No absorption enhancing effect was seen with the chosen concentration in this study, but a similar concentration has been reported to show an absorption enhancing effect for insulin over rabbit nasal tissue in vivo (Bechgaard et al., 1991). Monolayers incubated with a solution of HBSS containing mannitol to give a osmolality of about 700 mosm and with an isoosmotic (300 mosm) solution of HBSS served as

TABLE 1

Transport of mannitol and insulin through monolyaers of Caco-2 cells with or without addition of absorption enhancer

Experiment	n	Osmolality (mosm)	P_{app} ($\times 10^8$) (cm/s)	t-test [a]
Mannitol	4	– [c]	5.7 ± 1.0	–
+5.1 mM GC	4	274	5.2 ± 1.1	$p > 0.05$
+17.2 mM GC	4	293	154.1 ± 33.7	$p < 0.001$
+24.2 mM GC	4	297	685.4 ± 481.9	$p < 0.001$
+23.7 mM GF	4	296	6.1 ± 1.5	$p > 0.05$
+117 mM GF	4	386	3.9 ± 0.5	$p < 0.05$
+380 mM GF	3	666	8.9 ± 5.8	$p > 0.05$
Insulin	5	322	≤ 9.8 ± 10.0 [b]	–
+24.2 mM GC	2	350	266.5 ± 67.5	$p < 0.001$
+380 mM GF	5	697	12.7 ± 13.4	$p > 0.05$

[a] P_{app} was tested by two-sample t-test.

[b] Two out of five experiments showed absorption below the detection limit (3 μIU/ml). This limit is used in the calculation of means.

[c] Not determined.

P_{app} is expressed as mean ± S.D. (GC, sodium glycocholate; GF, glycofurol 75).

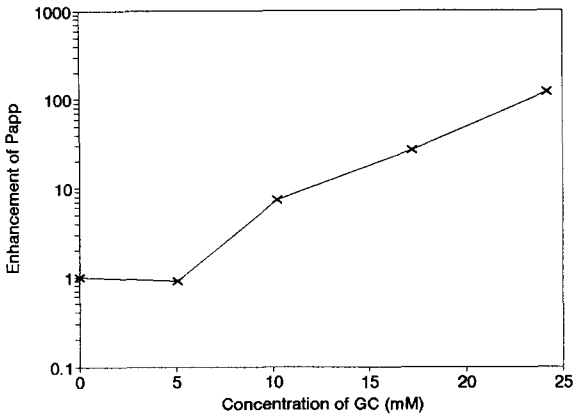

Fig. 2. Concentration dependent effect of sodium glycocholate (GC) on the permeability of mannitol through monolayers of Caco-2 cells.

controls. The formalin fixed specimens were consecutively placed in 1% osmium tetroxide and 1% uranyl acetate, dehydrated and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and examined with a Philips 420 electron microscope operated at 60 kV.

Results

Intracellular enzyme activity

A dose dependent decrease in dehydrogenase activity was seen for both GF and GC (Fig. 1). A small change in GC concentration in the range of 10–40 mM (0.5–2%) reduced the enzymatic activity dramatically, whereas much higher concentrations of GF were needed to obtain a similar effect (> 100 mM; ~ 1.75%), and the decrease in activity/mM was much lower. The IC_{50} values for GF and GC were 380 and 24.2 mM, respectively.

Absorption and recovery study

As shown in Table 1, P_{app} for mannitol in untreated control monolayers was 5.7×10^{-8} cm/s. Addition of 5.1 mM (0.25%) GC did not

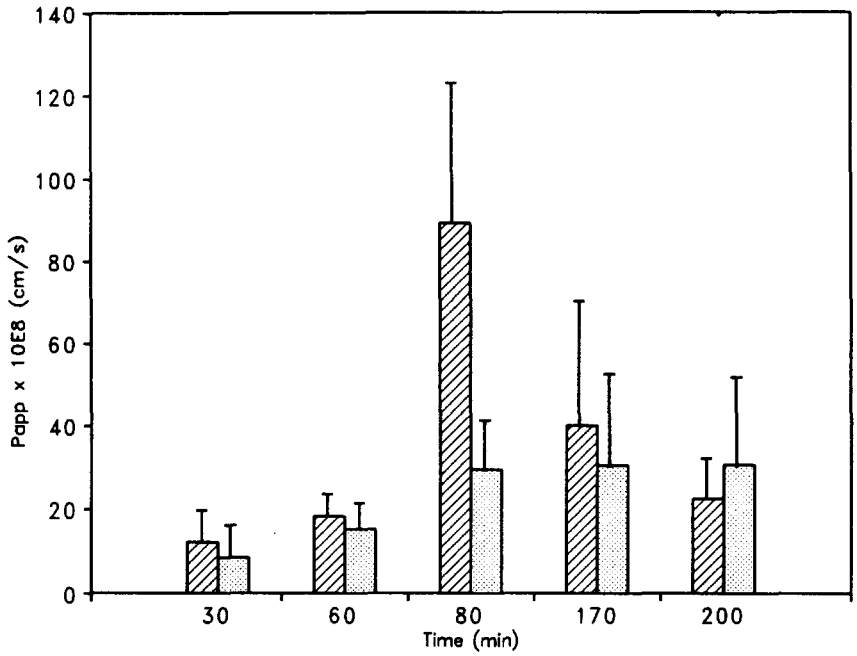

Fig. 3. Reversibility of the effect of sodium glycocholate (10.25 mM for 20 min) on mannitol absorption. The P_{app} values were determined before (30 and 60 min), during (80 min) and after (170 and 200 min) exposure to sodium glycocholate. The values are expressed as mean ± S.D. ($n = 4$). Hatched bars represent monolayers exposed to sodium glycocholate and stippled bars controls.

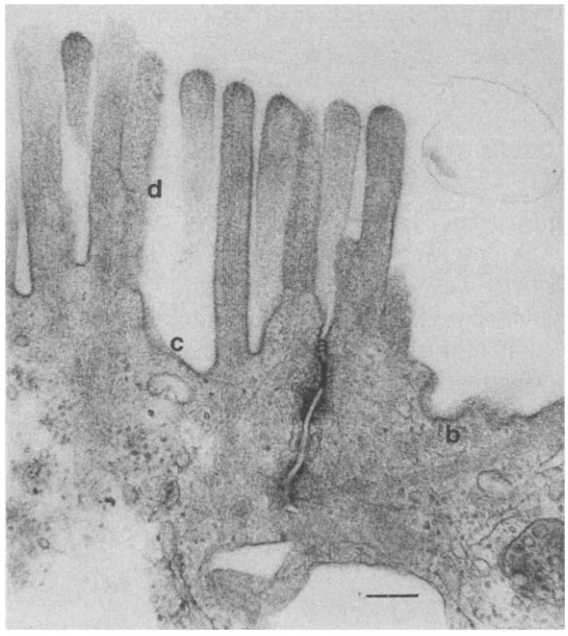

Fig. 4. Transmission electron micrograph of Caco-2 cells exposed to HBSS for 1 h. (a) Tight junction, (b) terminal web, (c) cell membrane, (d) microvilli. The bar indicates 0.2 μm.

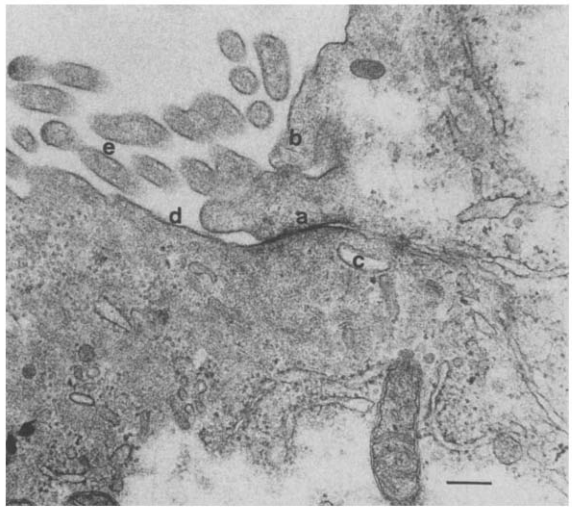

Fig. 5. Transmission electron micrograph of Caco-2 cells exposed to 380 mM ($\approx$ 6.8%) glycofurol 75 for 1 h. (a) Two cells joined by a tight junction with normal appearance. The cells have a disorganized terminal web (b), vacuoles (c), intact cell membrane (d) and disordered microvilli (e). The bar indicates 0.2 μm.

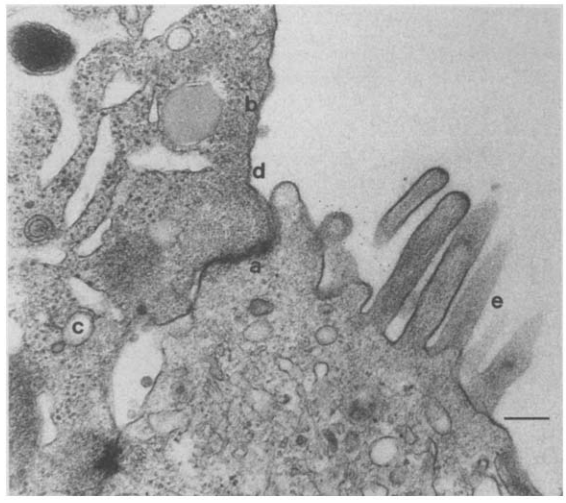

Fig. 6. Transmission electron micrograph of Caco-2 cells exposed to a mannitol solution with an osmolality of about 700 mosm for 1 h. The cells are joined by a tight junction (a). The terminal web is disorganized (b), vacuoles are obseved (c), the cell membrane is intact (d) and the microvilli disordered (e). The bar indicates 0.2 μm.

affect the P_{app}, whereas higher concentrations, 17.2 mM (0.8%) and 24.2 mM (1.2%), enhanced the absorption 27 and 120 times, respectively (Fig. 2). GF did not affect the absorption of mannitol significantly.

P_{app} for insulin (Table 1) was about the same as for mannitol ($\leqq 9.8 \times 10^{-8}$ cm/s), but P_{app} varied much more for insulin than for mannitol. Addition of GC (24.2 mM) affected the absorption by a factor of at least 22, whilst GF had no effect.

Fig. 3 shows the recovery of the permeability properties of the monolayer after exposure to 10.25 mM GC for 20 min. The monolayers recovered fully within 2 h after exposure. GC enhanced the P_{app} for mannitol by a factor of 7 (Fig. 3).

Transmission electron microscopy

TEM of the monolayers (Figs 4–6) showed that GF and a hyperosmotic mannitol solution affected the cells in a similar way. The treated Caco-2 cells showed disordered microvilli, a disorganized terminal web and many intracellular vacuoles, but no effects on the integrity of the

apical cell membrane and the tight junctions were observed.

Discussion

The use of absorption enhancers in drug formulation is complicated as most enhancers show some degree of toxicity. Concentrations of GC and GF which have shown absorption enhancing properties (Hirai et al., 1981a; Lee et al., 1988; Bechgaard et al., 1991) have in this study also been shown to inhibit intracellular dehydrogenase activity – a sign of cellular toxicity. Aungst et al. (1987) have used 102.5 mM (5%) GC to enhance absorption of insulin (nasal, rectal, buccal and sublingual), i.e., a concentration which resulted in complete inhibition of dehydrogenase activity in the present study. Most often, however, 20.5 mM (1%) GC is used (Hirai et al., 1981a; Duchateau et al., 1986; Lee et al., 1988). This concentration enhances the absorption significantly and corresponds to a concentration near the IC_{50} value.

Compared to GC, a more than 10 times higher concentration of GF is required to inhibit dehydrogenase activity. It is not known whether the inhibition caused by GF is due to the toxicity of GF or simply the result of the high osmolalities of the GF solutions. More detailed comparisons with hyperosmotic solutions of mannitol, which is not toxic, may provide the answer.

The permeability of insulin through monolayers of Caco-2 cells is comparable to insulin transport through other membranes. Schilling and Mitra (1990) have studied the transport of insulin across different intestinal segments from rats (everted sac) and found P_{app} values in the range of $0.8–7.8 \times 10^{-7}$ cm/s, where the duodenum showed the lowest and the distal jejunum the highest P_{app}. Lee et al. (1988) did not observe any transport of insulin across isolated rectal membranes of rabbits, however, 20.5 mM (1%) GC resulted in a P_{app} of 7×10^{-7} cm/s. The permeability of insulin across rabbit nasal mucosa in vitro has been determined to 4×10^{-7} cm/s (Bechgaard et al., 1992).

GC enhanced both mannitol and insulin transport. Low concentrations of GC did not enhance the absorption (Fig. 2), whereas concentrations above 5–10 mM showed an exponentiel rise in enhancement of absorption, i.e., an absorption enhancement is seen at concentrations near and above the threshold for toxicity as determined by the MTT test. At which concentration this rise in enhancement declines or ends was not studied. However, Lee et al. (1988) observed an increase in the effect of concentration up to at least 82.0 mM (4%) GC, in studies of insulin transport across isolated rectal membranes from rabbits.

Aungst et al. (1987) compared the efficacy of insulin by various routes of administration relative to i.m. They showed that the effect of GC on insulin efficacy depended on the route of administration. On addition of 102.5 mM (5%) GC, the efficacy of insulin was enhanced 117 times when administered nasally, but only 2.4 times when administered rectally. However, the rectal and nasal insulin doses were approximately equipotent after coadministration of GC. Hirai et al. (1981a) found that 20.5 mM (1%) GC enhanced the nasal bioavailability of insulin in rats by a factor of 6, which is comparable to the result in this study. This indicates that the method described by Anderberg et al. (1992) is useful for studying the effect of absorption enhancers on drug absorption.

In the present study, exposure to 10.25 mM (0.5%) GC resulted in a reversible absorption enhancement. This is in agreement with the results of Hirai et al. (1981b), who found that rat nasal mucosa recovered nearly completely within about 2 h after exposure to 20.5 mM (1%) GC. Thus, no difference in recovery time is observed, despite the difference in GC concentration. This is consistent with the findings of Anderberg et al. (1992) who showed that the Caco-2 monolayers are slightly more sensitive to sodium dodecyl sulfate and sodium dioctyl sulfosuccinate as compared to whole-tissue models.

These results indicate, that despite the difference between nasal and intestinal epithelium, studies of enhancers in the Caco-2 model may give useful information concerning nasal administration.

GF showed no improvement in absorption for either mannitol or insulin. This discrepancy from the in vivo observations may be due to the mechanism by which GF enhances absorption. Enhancers are believed to act by one or several mechanisms, e.g., inhibition of enzymatic degradation, alteration of thermodynamic activity of peptides, reduction of the viscosity of the mucus layer, reduction of the unstirred water layer, expansion of paracellular space, increase in membrane fluidity or reduction of peptide aggregation (Lee and Yamamoto, 1990).

The mechanism by which GF enhances insulin absorption in vivo is not through inhibition of enzymatic degradation, since Gizurarson and Bechgaard (1991) have shown that degradation of insulin is not limiting for intranasal application. The results in the Caco-2 model have excluded some of the proposed enhancer mechanisms, i.e., alteration of thermodynamic activity, expansion of paracellular space (cf. intact tight junctions (Fig. 5)), increased membrane fluidity and reduction of self-aggregation.

Karlsson and Artursson (1991) have shown that the aqueous boundary layer do not influence the permeability coefficient for mannitol and other less permeable drugs (i.e., insulin) under the conditions used in this study and the Caco-2 cell line do not produce mucus, i.e., an effect of GF at the mucus layer is not detectable in this model. This could explain the discrepancy between the observations in vivo and in vitro.

Morphological observations after exposure to hyperosmotic GF and mannitol solutions in this study are consistent with findings by Madara (1983) who studied morphological changes of the guinea pig jenunum after exposure to a hyperosmotic mannitol solution (600 mosm). These observations indicate that the effect of the GF solution is due to an osmotic effect and not to GF itself.

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