The Use of Surfactants to Enhance the Permeability of Peptides Through Caco-2 Cells by Inhibition of an Apically Polarized Efflux System

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Purpose. It has recently been reported that the permeability of peptides across Caco-2 cells, an *in vitro* model of the intestinal mucosa, was limited by an apically polarized efflux mechanism. Since surfactants (e.g. Cremophor EL, Polysorbate 80) have been reported to inhibit similar efflux systems in tumor cells, we determined whether they could enhance the permeability of peptides across monolayers of Caco-2 cells.

Methods. The transport studies of [^{3}H]-mannitol and [^{14}C]-model peptides were carried out across the Caco-2 cell monolayers. TEER values were determined using Voltohmmeter with STX-2 electrode and the equilibrium dialysis studies were conducted using side-by-side dialysis apparatus with cellulose ester membranes.

Results. Initially, [^{3}H]-mannitol flux studies were conducted to find concentrations of the surfactants that did not cause damage to the cell monolayer. Based on these studies, Polysorbate 80 and Cremophor EL were selected for further study. The fluxes of [^{14}C]-AcfNH$_2$ (a nonsubstrate for this efflux system) and [^{14}C]-Acf(N-Mef)$_2$NH$_2$ (a substrate for this efflux system) were then measured in the absence and presence of the two surfactants. The permeability of [^{14}C]-AcfNH$_2$ was not affected by the surfactants, while that of [^{14}C]-Acf(N-Mef)$_2$NH$_2$ increased with increasing concentrations of surfactants and then decreased. For example, the P_e values for [^{14}C]-Acf(N-Mef)$_2$NH$_2$ were 3.75×10^{-6}, 8.58×10^{-6}, 10.29×10^{-6}, 7.48×10^{-6}, and 1.46×10^{-6} cm/sec with Cremophor EL concentrations of 0, 0.01, 0.1, 1 and 10% w/v, respectively. This bimodal effect of surfactants on the Caco-2 cell permeability of this peptide was shown to be due to the interactions between the peptide and micelles at higher concentrations of surfactants, which were demonstrated by the equilibrium dialysis experiments.

Conclusions. These results suggest that surfactants, which are commonly added to pharmaceutical formulations, may enhance the intestinal absorption of some drugs by inhibiting this apically polarized efflux system.

KEY WORDS: Caco-2; polarized efflux system; Polysorbate 80; Cremophor EL; P-glycoprotein.

INTRODUCTION

The development of peptides as orally bioavailable therapeutic agents is limited by the poor permeability of these molecules across the intestinal mucosa. This poor intestinal permeability is in part due to the undesirable physicochemical properties (e.g. size, charge) of the peptides. However, it has recently been shown that peptides are also substrates for an apically polarized transport system that limits their permeability across a cell culture model of the intestinal mucosa (1). Similar efflux systems, of which the most well known is P-glycoprotein (2, 3) have been extensively studied because they are responsible for the drug resistance observed in multidrug resistant tumor cells.

P-glycoprotein has also been shown to exist in normal epithelial [e.g. intestinal mucosa (4)] and endothelial [e.g. blood brain barrier (5)] cells *in vivo* that represent barriers to drug delivery. In addition, the polarized expression and activity of a similar system has also been demonstrated in Caco-2 cells, an *in vitro* cell culture model of the intestinal mucosa (6, 7). Burton *et al.* (1) showed polarized transport of a model peptide in Caco-2 cells by a system with functional similarity to P-glycoprotein. Specifically, it was shown that [^{14}C]-AcfNH$_2$ is not a substrate for this apically polarized efflux system whereas [^{14}C]-Acf(N-Mef)$_2$NH$_2$ is a substrate for this system. Further, the transport could be saturated and competitively inhibited with verapamil.

Recently, medicinal chemists have become interested in the design and syntheses of potential inhibitors of P-glycoprotein as therapeutic agents to increase the efficacy of antitumor agents [i.e. to prevent the efflux of these antitumor agents in multidrug-resistant tumor cells (8, 9)]. However, such inhibitors could also potentially be used to enhance the intestinal absorption of drugs whose permeabilities are limited by an apically polarized efflux system. It is also possible that some excipients (e.g. surfactants), which are commonly added to pharmaceutical formulations, may function to enhance intestinal permeability of drugs by inhibiting this efflux mechanism. For example, it is well recognized that several surfactants (e.g. Polysorbate 80 (commonly known as Tween 80), Cremophor EL) are inhibitors of P-glycoprotein in cancer cells and thus enhance the uptake of antitumor agents (10).

Therefore, in this study we have examined the effect of the commonly used surfactants Polysorbate 80 and Cremophor EL on the transport of [^{14}C]-AcfNH$_2$ (a nonsubstrate for the efflux system) and [^{14}C]-Acf(N-Mef)$_2$NH$_2$ (a substrate for the efflux system) using the Caco-2 cell system. The results of these studies suggest that, along with their well recognized solubilizing activity, some surfactants commonly added to pharmaceutical formulations may also enhance absorption of drugs by inhibiting cellular efflux systems.

MATERIALS AND METHODS

Materials

[^{3}H]-Mannitol (specific activity, 50 μCi/nmol) was purchased from NEN Research Products, Du Pont Company (Wilmington, DE). [^{14}C]-AcfNH$_2$ and [^{14}C]-Acf(N-Mef)$_2$NH$_2$ (specific activity, 100 μCi/μmol) were prepared from the D-amino acids as previously described (11, 12). Triton X-100 (Lot # 122H0766), Nonidet P-40 (Lot # 12H2500) and CHAPS (Lot # 32H5029) were all purchased from Sigma Chemical Company (St. Louis, MO), while Cremophor EL (Lot # 32-1224) was obtained from BASF Corporation (Parsippany, NJ).

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ABBREVIATIONS: f, D-phenylalanine; CMC, critical micelle concentration.

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Polysorbate 80 (Lot # CF07429AF) was purchased from Aldrich Chemical Company (Milwaukee, WI) and Spectra/Por® cellulose acetate membranes for equilibrium dialysis experiments were obtained from Spectrum (Houston, TX). N-2-Hydroxyethylpiperazine-N′-2-ethane-sulfonate (Hepes), Hank's balanced salt solution (HBSS), Dulbecco's modified Eagles medium (DMEM), and non-essential amino acid (NEAA) were obtained from JRH Biosciences (Lenexa, KS). Fetal bovine serum (FBS) was from Intergen Company (Cambridge, MA). Rat tail collagen (Type I) was from Collaborative Research (Lexington, MA). Penicillin and streptomycin were obtained as a mixture from Irvine Scientific (Santa Ana, CA). Transwell® clusters, PVP free, 24.5 mm in diameter (4.71 cm^2 surface area), and 3.0 μm pore size were purchased from Costar Corporation (Bedford, MA). The Evom Epithelial Voltohmmeter and STX-2 electrode were bought from World Precision Instruments, Inc (New Haven, CT).

Cell Culture

The Caco-2 cell line, originating from a human carcinoma (13), was obtained from American Type Culture Collection (Rockville, MD) and was grown as described previously (14). Briefly, cells were grown in 165 cm^2 culture flasks (Costar Corp.) in culture medium consisting of DMEM with 100 U/mL penicillin and 100 μg/mL streptomycin, 1% NEAA and 10% FBS. Before reaching confluency, cells were trypsinized with 0.25% trypsin and 0.02% EDTA and plated at a density of 63,000 cells/cm^2 in a culture medium on Transwell® polycarbonate membranes (3.0 μm pore size), which had previously been coated with collagen. The culture medium was replaced [1.5 mL apical (AP) side and 2.6 mL basolateral (BL) side] every other day for the first week and daily thereafter. Cells were maintained at 37°C in an atmosphere of 5% CO_2 and 95% relative humidity. All cells used in this study were between passages 50 and 60.

Transport Studies

All transport experiments were performed for 2 hours at 37°C in pH 7.35 HBSS containing 25 mM glucose and 10 mM Hepes buffer (transport medium) unless stated otherwise in the figure legends. Prior to the experiments, the culture medium of Transwell®-grown Caco-2 cell monolayers was replaced with transport medium equilibrated at 37°C, and the cell monolayer was subsequently equilibrated for 30 minutes at 37°C before undertaking the transport studies. In AP-to-BL transport studies, all wells in six-well clusters received 2.6 mL of transport medium that had previously been equilibrated at 37°C. Inserts containing the Caco-2 cell monolayers were positioned in the wells such that the outer surface of the inserts (BL side) was immersed in the transport medium. Transport medium (1.5 mL) containing the relevant substance of which transport properties were to be determined, with or without surfactant, was then applied to the AP side. At indicated time intervals prior to sampling, the six-well cluster cell culture plate was gently swirled to ensure complete mixing. Samples (100 μL) were then withdrawn carefully from the BL side and replaced with the same volume of fresh transport medium.

In BL-to-AP transport studies, inserts were positioned in the wells containing 2.6 mL of the [^{14}C]-Acf(N-Mef)$_2$NH$_2$-containing solutions. Transport medium containing surfactant was then applied on the AP side. At indicated time intervals prior to sampling, the six-well cluster cell culture plate was gently swirled to ensure complete mixing. Samples (100 μL) were then withdrawn carefully from the AP side and replaced with the same volume of fresh surfactant solution. The amount of radioactive material transported was determined in a Beckman LS 6000 IC liquid scintillation counter.

Permeability coefficients (P_e) were calculated using the following equation:

$$P_e = \frac{1}{ACo} \cdot \frac{dQ}{dt}$$

where dQ/dt is the flux across the monolayer (DPM/min), A is the surface area of the membrane (4.71 cm^2) and Co is the initial drug concentration (DPM/mL).

Determination of Transepithelial Electrical Resistance (TEER) Values

The cells were checked for confluency by measurement of TEER values first in HBSS and then after treating the monolayers with different surfactant concentrations for 2 hours using the Evom Epithelial Voltohmmeter and STX-2 electrode.

Equilibrium Dialysis Experiments

A Spectrum side-by-side equilibrium dialysis apparatus was used with Spectra/Por® cellulose acetate membranes (molecular weight cut-off 1000 Da) between the two compartments. The donor compartment was filled with 1 mL of peptide solution (1 μM; specific activity, 100 μCi/μmol) with or without surfactant, while the receiver compartment was filled with an equal volume of HBSS buffer. During the control experiment (i.e. without any surfactant added to the donor compartment), the samples were withdrawn from the donor and receiver compartments to determine the time needed for the system to reach equilibrium. The sampling time points for all subsequent experiments were then determined from these control experiments. This ensures that the sampling is done after the system reaches equilibrium.

Statistical Analysis

All data were analyzed by analysis of variance.

RESULTS

Effect of Surfactants on [^{3}H]-Mannitol Flux and TEER Values

The effects of various surfactants at several different concentrations on the AP-to-BL transport of [^{3}H]-mannitol in HBSS buffer were determined. These surfactants included Polysorbate 80, Cremophor EL, Triton X-100 and Nonidet P-40, which are nonionic surfactants, and CHAPS, which is a zwitterionic surfactant. The results shown in Table I suggest that Polysorbate 80 in the concentration range of 0.01–1% w/v and Cremophor EL in the range of 0.01–10% w/v produced no functional damage to the cell monolayer as measured by [^{3}H]-mannitol flux. In contrast, CHAPS, Triton X-100 and Nonidet P-40 caused significant increases in [^{3}H]-mannitol flux, which indicated

Table I. Concentration-dependent Effect of Various Surfactants on the AP-to-BL Transport of [^{3}H]-Mannitol

Surfactant	$P_e \times 10^6$ (cm/sec) of [^{3}H]-mannitol					
	0% w/v	0.01% w/v	0.05% w/v	0.1% w/v	1% w/v	10% w/v
None	0.28 (0.06)					
Polysorbate 80		0.27 (0.05)	0.25 (0.04)	0.25 (0.03)	0.21 (0.06)	ND**
Cremophor EL		0.27 (0.06)	ND	0.38 (0.05)	0.26 (0.02)	0.27 (0.06)
CHAPS		0.39 (0.08)	0.29 (0.08)	0.26 (0.01)	20.87 (1.62)*	ND
Nonidet P-40		6.19 (1.22)*	10.79 (1.34)*	11.5 (3.24)*	16.99 (1.06)*	ND
Triton X-100		0.31 (0.10)	11.50 (0.31)*	12.38 (3.53)*	21.58 (4.25)*	ND

21-day-old Caco-2 cell monolayers were treated on the AP side with 1.5 mL of transport medium containing 8.8 pmol/mL of [^{3}H]-mannitol (specific activity, 50 μCi/nmol) with different surfactants (e.g. Polysorbate 80, Cremophor EL, CHAPS, Nonidet P-40, Triton X-100) in the concentration ranges shown. At various time intervals, samples (100 μL) were removed from the BL side. The transport experiments were carried out for 3 hours. Permeability Coefficients (P_e) of [^{3}H]-mannitol were then calculated based on the flux values obtained from these experiments. The values in parentheses represent standard deviation for three experiments. Statistically significant differences were estimated using analysis of variance: *$P < 0.05$ versus control. ** ND = not determined.

that these surfactants damage the cell monolayers. To further confirm that Polysorbate 80 (0.01–1% w/v) and Cremophor EL (0.01–10% w/v) were not damaging the cell monolayers, the effects of these surfactants on the TEER values were determined. When these surfactants were applied to the AP side of the cell monolayer for two hours at the concentrations mentioned above, no changes in TEER values were observed (data not shown). In light of these observations, Polysorbate 80 up to the highest concentration of 1% w/v and Cremophor EL up to the highest concentration of 10% w/v were selected for subsequent experiments.

Effect of Polysorbate 80 and Cremophor EL on the AP-to-BL and BL-to-AP Transport of [^{14}C]-AcfNH$_2$ and [^{14}C]-Acf(N-Mef)$_2$NH$_2$

Table II shows the P_e values of [^{14}C]-AcfNH$_2$ and [^{14}C]-Acf(N-Mef)$_2$NH$_2$ in the AP-to-BL direction in the absence and presence of various concentrations of Polysorbate 80 and Cremophor EL. Even at the highest concentrations employed in this study, the two surfactants did not change the AP-to-BL P_e value of [^{14}C]-AcfNH$_2$ compared to the P_e value determined in HBSS. In contrast, a bimodal effect was observed in the presence of Polysorbate 80 and Cremophor EL in the case of [^{14}C]-Acf(N-Mef)$_2$NH$_2$ permeability measured in the AP-to-BL direction. With increasing concentrations of Polysorbate 80, the P_e value of [^{14}C]-Acf(N-Mef)$_2$NH$_2$ increased initially and then reached a plateau level at high concentrations of surfactant. In the case of Cremophor EL, the P_e value of [^{14}C]-Acf(N-Mef)$_2$NH$_2$ increased initially, followed by a decrease in permeability at high concentrations of surfactant.

When the permeability of the [^{14}C]-Acf(N-Mef)$_2$NH$_2$ was studied in the BL-to-AP direction with the surfactant placed on the AP side, the P_e values of the peptide decreased with increasing surfactant concentrations, eventually reaching a plateau level (Table II). A bimodal effect of the type observed above for the AP-to-BL flux of this peptide was not observed for the BL-to-AP flux data.

Effect of Varying Concentrations of Surfactants on the Fractions of [^{14}C]-AcfNH$_2$ and [^{14}C]-Acf(N-Mef)$_2$NH$_2$ Bound to Micelles

In order to determine the interaction between the model peptides and surfactant micelles, the fraction of peptide bound to micelles was estimated from equilibrium dialysis experiments. The results shown in Fig. 1 indicate that the interaction of [^{14}C]-AcfNH$_2$ is minimal until very high concentrations of surfactants are introduced. In contrast, in the case of [^{14}C]-Acf(N-Mef)$_2$NH$_2$, there is a significant increase in the fraction of peptide bound to micelles with increasing concentrations of both Polysorbate 80 and Cremophor EL.

DISCUSSION

Caco-2 cells, a human adenocarcinoma cell line, have been shown to be a valuable *in vitro* model of the intestinal mucosa (14–16). When grown onto polycarbonate membranes, these cells undergo differentiation, ultimately exhibiting morphological and biochemical characteristics similar to those observed in villus epithelial cells (14). One biochemical characteristic exhibited by Caco-2 cells, which is of particular interest in this study, is the expression of an apically polarized efflux system (6). This efflux system has been shown to limit the Caco-2 permeability of some solutes (1, 7) and is thus functionally (and probably structurally) similar to the P-glycoprotein mediated efflux system observed in multidrug resistant tumor cells (2, 3).

In recent years, medicinal chemists have designed and synthesized many novel inhibitors of P-glycoprotein in tumor cells as potential therapeutic agents (8, 9). However, it is also well recognized that surfactants (e.g. Polysorbate 80, Cremophor EL) are inhibitors of this efflux system in cancer cells (17). For example, Friche *et al.* (18) have reported that Polysorbate 80 and Cremophor EL inhibit labeling of P-glycoprotein by [^{3}H]-azidopine in EHR2 (wild type Ehrlich ascites tumor) cells. We were particularly interested in these observations, as several of these surfactants are commonly used in pharmaceutical formulations. Thus, the beneficial effects (e.g. increased oral bioavailability) of including a surfactant in a pharmaceutical formulation may be in part due to the ability of the surfactants to inhibit these efflux systems in intestinal mucosal cells. Therefore, we examined the effects of these commonly used surfactants on the efflux system in Caco-2 cells.

Surfactants, which are amphiphilic molecules, are known to cause changes in the fluidity of the cell membranes and also to cause cell damage at high concentrations (19). Therefore, it was essential to determine a concentration range for each

Table II. AP-to-BL Transport of [^{14}C]-AcfNH$_2$ and [^{14}C]-Acf(N-Mef)$_2$NH$_2$ and BL-to-AP Transport of [^{14}C]-Acf(N-Mef)$_2$NH$_2$ in the Absence and Presence of Various Concentrations of Polysorbate 80 and Cremophor EL

	$P_e \times 10^6$ (cm/sec)		
Surfactant (% w/v)	AP-to-BL AcfNH$_2$	AP-to-BL Acf(N-Mef)$_2$NH$_2$	BL-to-AP Acf(N-Mef)$_2$NH$_2$
None	6.98 (0.67)	3.85 (1.44)	34.31 (1.92)
Polysorbate 80 (0.0001)	6.31 (0.47)	5.14 (1.62)	34.32 (1.59)
(0.0005)	6.61 (1.76)	5.62 (0.92)	26.52 (3.69)
(0.001)	6.74 (0.66)	7.44 (0.56)	23.92 (1.99)
(0.005)	6.68 (0.52)	8.58 (0.48)	20.11 (1.01)
(0.05)	7.50 (0.83)	9.44 (1.25)	15.82 (1.79)
(0.1)	7.73 (0.72)	8.95 (0.95)	15.94 (0.52)
(1)	8.49 (1.83)	8.28 (0.45)	13.83 (2.61)
Cremophor EL (0.0001)	7.01 (0.93)	4.67 (0.37)	30.76 (5.31)
(0.001)	6.21 (0.33)	6.26 (0.89)	25.69 (1.01)
(0.005)	5.52 (0.21)	8.33 (1.12)	19.50 (0.75)
(0.01)	6.79 (0.09)	8.59 (1.03)	17.48 (0.80)
(0.1)	6.93 (0.94)	10.05 (1.59)	13.43 (2.47)
(1)	7.36 (0.63)	7.41 (0.40)	15.76 (0.91)
(10)	5.92 (0.39)	1.50 (0.06)	15.70 (1.49)

Note: For the AP-to-BL transport, 21-day old Caco-2 cell monolayers were treated on the AP side with 1.5 mL of transport medium containing 1 μM of [^{14}C]-AcfNH$_2$ or [^{14}C]-Acf(N-Mef)$_2$NH$_2$ (specific activity, 100 μCi/μmol) in the absence and presence of Polysorbate 80 and Cremophor EL in the concentration ranges shown. At 15 minutes time intervals, samples (100 μL) were removed from the BL side. For the BL-to-AP transport, 21-day-old Caco-2 cell monolayers were treated on the AP side with 1.5 mL of surfactant solution and placed in the wells with 2.6 mL of transport medium containing 1 μM of [^{14}C]-Acf(N-Mef)$_2$NH$_2$ (specific activity, 100 μCi/μmol). At various time points, samples (100 μL) were removed from the AP side. The P_e were then calculated based on the flux values obtained from these experiments. Values in parentheses represent standard deviation for three experiments.

surfactant that did not cause damage to the Caco-2 cell monolayers. [^{3}H]-Mannitol P_e values and TEER values were used to determine whether these surfactants caused damage to the cell monolayers. The results of these experiments, which are shown in Table I, demonstrated that Polysorbate 80 up to 1% w/v and Cremophor EL up to 10% w/v did not cause any apparent damage to the cell monolayers within the experimental period of exposure. These observations are consistent with the reports by Woodcock *et al.* (17) that Polysorbate 80 in 1% w/v and Cremophor EL up to 10% w/v did not cause lysis of human leukemic R 100 cells. Based on the results shown in Table I, Polysorbate 80 and Cremophor EL were selected for further study.

Burton *et al.* (1) have reported a significant difference in the transport characteristics of [^{14}C]-AcfNH$_2$ and [^{14}C]-Acf(N-Mef)$_2$NH$_2$ in Caco-2 cells. For [^{14}C]-Acf(N-Mef)$_2$NH$_2$, the AP-to-BL permeability was significantly lower than that of the BL-to-AP permeability at low peptide concentration. Further, increasing the peptide concentration or inclusion of verapamil, a known inhibitor of P-glycoprotein, increased AP-to-BL flux and decreased BL-to-AP flux until a common permeability was achieved. In contrast, when the AP-to-BL flux of [^{14}C]-AcfNH$_2$ was compared to its BL-to-AP flux, no significant difference was observed. In addition, verapamil had no significant effect on either the AP-to-BL or the BL-to-AP flux of this modified amino acid. These results suggest that [^{14}C]-AcfNH$_2$ is permeating the cell monolayer strictly by passive diffusion whereas the permeability of [^{14}C]-Acf(N-Mef)$_2$NH$_2$ is being modified by an apically polarized efflux system that is saturable, substrate-specific and inhibitable by known inhibitors of P-glycoprotein (1). Therefore, for these studies we chose [^{14}C]-AcfNH$_2$ as a nonsubstrate for the efflux system and [^{14}C]-Acf(N-Mef)$_2$NH$_2$ as a substrate.

As shown in Table II, the AP-to-BL permeability of [^{14}C]-Acf(N-Mef)$_2$NH$_2$ increased, whereas the BL-to-AP permeability of this tetrapeptide decreased with increasing concentrations of either Polysorbate 80 or Cremophor EL applied on the AP side of the cell monolayer. In contrast, these surfactants had no effect on the AP-to-BL flux of [^{14}C]-AcfNH$_2$. These results suggest that the surfactants are acting like verapamil, causing the inhibition of the apically polarized efflux system in Caco-2 cells.

The decrease in AP-to-BL permeability of [^{14}C]-Acf(N-Mef)$_2$NH$_2$ at higher surfactant concentrations can be explained on the basis of the high fraction of [^{14}C]-Acf(N-Mef)$_2$NH$_2$ bound to the surfactant micelles (Fig. 1). At high concentrations of Polysorbate 80 and Cremophor EL, there is a significant interaction between the micelles and [^{14}C]-Acf(N-Mef)$_2$NH$_2$, which decreases the concentration of "free" peptide on the AP (donor) side of the cell monolayers. Since the permeability coefficients are calculated from the flux of free solute divided by the total peptide concentration, which reflects both free and bound species, the apparent permeabilities decrease. Further, since the non-passive, polarized efflux pathway is also concentration dependent, decreasing the free concentration of peptide will result in a net increase in the resistance to transport in the AP-to-BL direction. Thus, the observed permeability will represent the relative contributions of these two competing phenomena. However, a similar interaction between [^{14}C]-AcfNH$_2$ and micelles was not observed (Fig. 1) and, hence, its permeability does not change appreciably at high surfactant concentration (Table II).

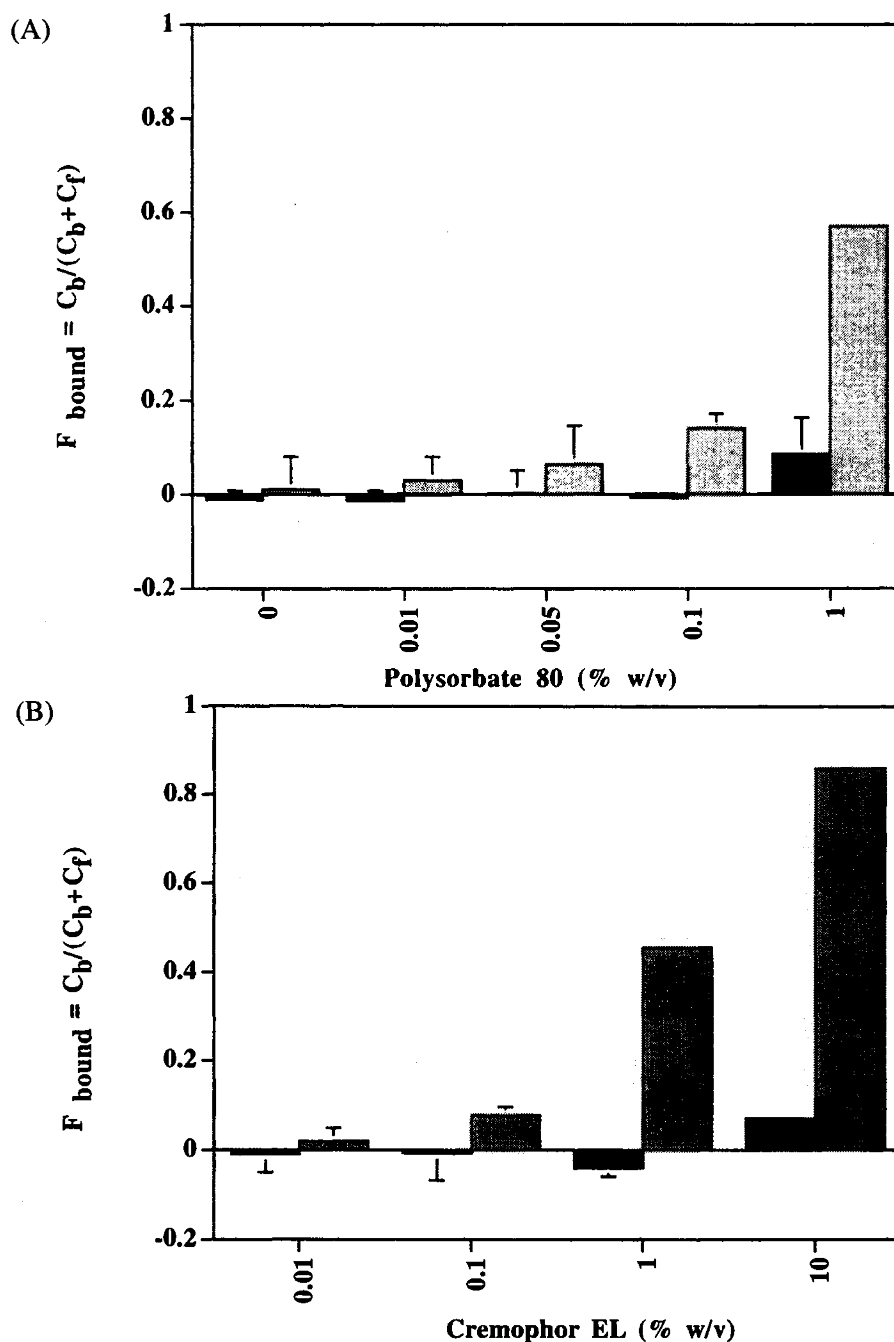

Fig. 1. Effects of increasing concentrations of Polysorbate 80 and Cremophor EL on the fractions of [^{14}C]-AcfNH$_2$ and [^{14}C]-Acf(N-Mef)$_2$NH$_2$ bound (F_{bound}) to the micelles. A side-by-side equilibrium dialysis apparatus was used with Spectra/Por® cellulose acetate membranes (molecular weight cut-off 1000) between the two compartments. The donor compartment was filled with a 1 mL solution of [^{14}C]-AcfNH$_2$ (■) or [^{14}C]-Acf(N-Mef)$_2$NH$_2$ (■) at a final concentration of 1 μM (specific activity, 100 μCi/μmol) with Polysorbate 80 (panel A) or Cremophor EL (panel B) or without surfactant, while the receiver compartment was filled with an equal volume of HBSS buffer. At equilibrium, samples from both donor and receiver compartments were withdrawn and the fraction of peptide bound to micelles was calculated by dividing the concentration of bound peptide by the total peptide concentration.

In contrast, the decrease in permeability of [^{14}C]-Acf(N-Mef)$_2$NH$_2$ in the BL-to-AP direction in the presence of Polysorbate 80 or Cremophor EL as compared to the control value (Table II) is of particular importance since this experimental design eliminates any peptide-surfactant interactions that are present in the AP-to-BL transport experiments (Table II). Based on the preliminary studies, where surfactants in their increasing concentrations were added on the AP side and the changes in the surface tension of the BL side solution were monitored, it was concluded that the surfactants are not capable of crossing the cell monolayers as no significant changes in surface tension as a function of increasing surfactant concentrations were observed (data not shown). Unlike the AP-to-BL transport studies, where peptide and surfactant were present on the same side, the BL-to-AP transport experiments had surfactant molecules only on the AP side, whereas the donor peptide solution was present on the BL side. Given that the surfactants themselves are incapable of crossing the cell monolayers, this allows the surfactant molecules access to the apically polarized efflux system without any interference from the peptide-surfactant interactions (pre- and post-micellar interactions). In this case, BL-to-AP permeability decreased with increasing surfactant concentration until a plateau was reached, unaffected by further increases in surfactant concentrations (Table II). These results are consistent with those seen with verapamil inhibition of this transport system (20) and further support the apical membrane location of the transporter in Caco-2 cells.

With respect to the mechanism by which these surfactants inhibit this efflux system, nonionic surfactants such as Polysorbate 80 and Cremophor EL are shown to integrate within the cell membranes (21) and thus change their microviscosity (18). This results in the loosening of the phospholipid bilayers of cells, resulting in the loss of secondary and/or tertiary structures of membrane proteins, thus altering their biological activity. This may be the mechanism by which the surfactants inhibit this efflux system. However, it has also been reported that the zwitterionic surfactant CHAPS, which can integrate within the phospholipid bilayers, does not show an inhibition of P-glycoprotein even at high concentrations (17, 21). Hence the observed differences between nonionic and zwitterionic surfactants in inhibition of P-glycoprotein may be due to the differences in the binding affinities of these surfactants to the hydrophobic portion of this efflux system (21). This, in turn, means that the inhibition of this efflux system by nonionic surfactants such as Polysorbate 80 and Cremophor EL may have a component of specific binding to the hydrophobic domain of the protein along with the non-specific changes in its secondary and/or tertiary structure of the protein.

On the other hand, the observation that, for both Polysorbate 80 and Cremophor EL, increasing surfactant concentration beyond the CMC (~0.005% and 0.0095% w/v respectively) results in the continued reduction in BL-to-AP permeability for Acf(N-Mef)$_2$NH$_2$ suggests that along with monomer, surfactant micelle itself is also active as an inhibitor. In this case, rather than interact with the cell membrane directly, the micelle may solubilize the specific membrane components and influence protein structure and function indirectly through changes in local membrane composition. Further experiments are needed to sort out these mechanistic possibilities.

While these results provide additional evidence for an apically polarized efflux system in Caco-2 cells, they do not identify the protein(s) responsible for this effect. The literature would suggest that this effect is probably mediated by a member of the P-glycoprotein family. This family of proteins is responsible for the multidrug resistance in cancer cells (22). However, P-glycoproteins, which are 170–180 KDa membrane glycoproteins, have also been observed to be present in the apical region of several epithelia (e.g. small intestine, colon, liver, kidney) (4) and endothelia (e.g. brain endothelial cells) (5). This family of proteins serves as an ATP-dependent membrane efflux pump, thereby reducing the intracellular accumulation or transcellular flux of a wide variety of drugs.

In summary, these studies have shown the participation of surfactants in blocking an apically polarized efflux system and increasing the transport of a peptide that is a substrate for this efflux pump. Hence, apart from their use in pharmaceutical formulations as excipients, surfactants can also be used to enhance the intestinal permeability of drugs by inhibition of this apically polarized efflux system.

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