

Effects of Pluronic P85 Unimers and Micelles on Drug Permeability in Polarized BBMEC and Caco-2 Cells

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Received May 6, 1998; accepted June 25, 1998

Purpose. Using polarized bovine brain microvessel endothelial cells (BBMEC) monolayers as *in vitro* model of the blood brain barrier and Caco-2 monolayers as a model of the intestinal epithelium, the present work investigates the effects of Pluronic P85 block copolymer (P85) on the transport of the P-glycoprotein (P-gp)-dependent probe, rhodamine 123 (R123).

Methods. The permeability and cell efflux studies are performed with the confluent cell monolayers using Side-Bi-Side diffusion cells.

Results. At concentrations below the critical micelle concentration, P85 inhibits P-gp efflux systems of the BBMEC and Caco-2 cell monolayers resulting in an increase in the apical to basolateral permeability of R123. In contrast, at high concentrations of P85 the drug incorporates into the micelles, enters the cells and is then recycled back out to the apical side resulting in decrease in R123 transport across the cell monolayers. Apical to basolateral permeability of micelle-incorporated R123 in BBMEC monolayers was increased by prior conjugation of P85 with insulin, suggesting that modified micelles undergo receptor-mediated transcytosis.

Conclusions. Pluronic block copolymers can increase membrane transport and transcellular permeability in brain microvessel endothelial cells and intestinal epithelium cells. This suggests that these block copolymers may be useful in designing formulations to increase brain and oral absorption of select drugs.

KEY WORDS: pluronic block copolymer; intestinal delivery; drug; micelles; blood brain barrier.

INTRODUCTION

Amphiphilic block copolymers are of interest as potential drug delivery and drug targeting systems due to their ability to self-assemble into micelles and incorporate many drugs in the hydrophobic core (1–6). We have designed “microcontainers” for drug delivery using Pluronic block copolymers (poly(ethylene oxide)-*block*-poly(propylene oxide)-*block*-poly(ethylene oxide), EO_{m/2}-PO_n-EO_{m/2}) (2,7). After conjugation with a brain-specific antibody or insulin these microcontainers were shown to target haloperidol and a fluorescent marker to the brain of mice (2,7). In addition to the micellar delivery route recent studies have suggested that Pluronic single chains (“unimers”) can act as biological response modifiers by affecting certain drug transport systems (8,9). Studies on multiple drug resistant cancers have demonstrated that Pluronic unimers inhibited glycoprotein P (P-gp) efflux pump thus enhancing drug accumulation in these cells (8,9).

Combination of the Pluronic micellar delivery with the biological response modifying activity of the Pluronic unimers permits affecting drug transport in cells through multiple mechanisms. Using bovine brain microvessel endothelial cells (BBMEC) we have demonstrated that Pluronic P85 (P85) unimers increase drug uptake by inhibiting the P-gp efflux system (10). In contrast, the P85 micelles induce transient drug accumulation in these cells, which apparently involves enhanced vesicular transport (10). Very similar transport phenomena with Pluronic block copolymers were reported also in human colonic epithelium cells, Caco-2 (11).

Using the polarized BBMEC monolayers as *in vitro* model of blood brain barrier (BBB) and Caco-2 monolayers as a model of intestinal epithelium the present work investigates the effects of P85 on drug transport. The P-gp dependent probe, rhodamine 123 (R123), was used as a model drug to evaluate the effects of the P85 block copolymer on the efflux system in these cell models. This study for the first time suggests that both the unimer and micelle-mediated transport mechanisms can be used to affect the permeability of R123 across intestinal barrier and BBB. These findings may be useful for developing drug formulations to increase brain and oral absorption of select drugs.

MATERIALS AND METHODS

Cell Culture

BBMEC were isolated from fresh cow brains using a combination of enzymatic digestion and density centrifugation as described previously (12). R.T. Borchardt (The University of Kansas, Lawrence, KS) kindly provided Caco-2 cells, originating from a human colorectal carcinoma (13). The BBMEC cells were maintained in MEM:F12 culture medium supplemented with 10% horse serum, heparin sulfate (100 µg/mL), amphotericin B (2.5 µg/mL), and gentamicin (50 µg/mL). Caco-2 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM), containing 10% heat-inactivated fetal bovine serum (FBS), 1% non-essential amino acids, benzylpenicillin (100 U/ml) and streptomycin (10 µg/ml) as described elsewhere (14). All tissue culture media were obtained from Gibco Life Technologies, Inc. (Grand Island, NY). Cell monolayers were grown on collagen coated (Caco-2) or fibronectin and collagen coated (BBMEC) polycarbonate membrane inserts (Transwell, Costar Brand Tissue Culture Products, Contd.; pore size 0.4 µm; diameter 24.5 mm). Cells were seeded at a density of 250,000 cells per insert and were allowed to grow and differentiate for up to 14 days till complete maturation of the monolayers.

Preparation of Surfactant and Pluronic Block Copolymer Solutions

The present study uses P85 block copolymer (lot #WPOP-587A) that was provided by BASF Corp. (Parispany, NJ). The molecular mass of the PO segment in this sample was approximately 2,500 and the content of EO chains was approximately 50% (w/w). The solutions of P85 were prepared in assay buffer containing: 122 mM sodium chloride, 25 mM sodium bicarbonate, 10 mM glucose, 10 mM HEPES, 3 mM potassium chloride, 1.2 mM magnesium sulfate, calcium chloride (1.4 mM) and

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potassium phosphate dibasic (0.4 mM). R123 was added to either 22 μ M P85 (0.01% w/v), 2.2 mM P85 (1% w/v) or 11 mM P85 (5% w/v) solutions and incubated at 37°C for at least one hour prior to use in the experiments.

Synthesis of Ins-P85 Conjugate

One gram of P85 was reacted overnight at room temperature with 8 mg of 1,1'-carbonyldiimidazole (Sigma-Aldrich, St Louis, MO) in 2 ml of anhydrous acetonitrile, then dialyzed against 20% aqueous ethanol and lyophilized. At the second stage 200 mg of activated P85 was reacted with 120 mg of insulin (Ins) (Sigma-Aldrich, St Louis, MO) in 5.2 ml of borate buffer, pH 8.5 for 72 h at 4°C. The reaction mixture was dialyzed against 4 ml ammonium in 4 L water and the Ins-P85 conjugate was purified by gel permeation HPLC using SigmaChrom™ GFC-100 column (30 cm \times 7.5 mm) and 0.5 ml/min flow rate.

R123 Transport Studies

Polycarbonate membrane inserts with confluent BBMEC or Caco-2 monolayers were placed in Side-Bi-Side diffusion cells from Crown Bio Scientific, Inc. (Somerville, NJ) maintained at 37°C \pm 0.1°C. In apical (AP) to basolateral (BL) transport studies the AP side of the monolayers was exposed to the donor chamber, while in BL to AP studies the BL side of the membranes was exposed to the donor chamber. Cell monolayers were preincubated for 30 minutes at 37°C with the assay buffer, added to both donor and receiver chambers (3 ml). Following the pre-incubation period, fresh assay buffer was added to the receiver compartment; the assay buffer in the donor chamber was replaced with 3.2 μ M R123 in (i) assay buffer (control groups), (ii) P85 unimers (22 μ M P85 solution), or (iii) P85 micelles (2.2 mM or 11 mM P85 solution). In the experiments evaluating the effects of Ins on the micelle transport the following systems were used (i) 2.2 mM P85 and 1 nM Ins-P85 conjugate, (ii) (i) and 17 nM Ins, (iii) 2.2 mM P85 and 1 nM Ins. To account for the integrity of the monolayers the solutions in the donor chamber also contained H³-labeled mannitol, a paracellular marker (15), obtained from DuPont Corp. (Boston, NA). At 0.5, 15, 30, 60 and 90 min time points the solutions in the receiver chamber were removed for R123 determination using a Shimadzu RF5000 fluorescent spectrophotometer (λ_{ex} = 505 nm, λ_{em} = 540 nm) and H³-mannitol determination using a liquid scintillation counter (Hewlett Packard Instruments). Immediately after collecting the solutions in the receiver chamber 3 ml of fresh assay buffer was added to this chamber. The transport of R123 (or mannitol) across the cell monolayers was expressed as the percentage of the initial amount of R123 (or mannitol) in the donor chamber, that accumulated in the receiver chamber. A minimum of three different membranes was studied for each drug composition at multiple time points for each membrane. In certain BL to AP transport experiments evaluating the effects of P85 unimers 3.2 μ M R123 (and H³-labeled mannitol) in assay buffer was placed in the donor chamber, while 22 μ M P85 in assay buffer was placed in the receiver chamber, i.e. at the AP side of the monolayers. In these experiments fresh 22 μ M P85 in assay buffer was replaced for solutions removed from the receiver chamber for the probe determination.

R123 Efflux Studies

Confluent BBMEC or Caco-2 monolayers grown on membrane inserts were placed in the Side-Bi-Side diffusion cells. The cells were preincubated for 30 min at 37°C with assay buffer and then exposed to a loading solution of 3.2 μ M R123 in assay buffer for 60 min at 37°C from both the donor and receiver chambers. After that the solutions from donor and receiver chambers were removed and the cell monolayers were washed quickly with ice-cold PBS three times. The assay buffer or P85 micelle solution (2.2 mM P85 with BBMEC, 11 mM P85 with Caco-2) was added to the AP or BL side. At various time intervals the amounts of R123 effluxed from the cells monolayers was determined by sampling both the AP and BL compartments. The efflux of R123 was expressed on a percentage basis as the ratio of the total R123 accumulated in the respective chamber to the initial amount of R123 in the loading solution.

Fluorescence Microscopy

In separate studies, BBMEC were grown on collagen-coated, fibronectin-treated chamber slides (Fisher, St. Louis, MO) for examining R123 cellular accumulation using fluorescent microscopy. The BBMEC were exposed to the R123 in 2.2 mM P85 solutions for 5, 15, 30, and 90 minutes at 37°C. After this incubation period, the loading solutions were removed and the BBMEC were washed a total of three times with ice-cold PBS and examined using a Leitz fluorescent microscope.

RESULTS

Effects of P85 Unimers on R123 Flux Across Cell Monolayers

Our previous studies have demonstrated that P85 single chains (unimers) increase accumulation of the P-gp substrate, R123, in BBMEC (10) and Caco-2 (11) monolayers. The specificity of the effect of the unimers on the P-gp efflux systems in both cell models was evidenced by the absence of the P85 unimer effects on the transport of the non-P-gp dependent analog of R123, rhodamine 110 (10,11). In the present study we evaluate whether the transport of R123 across the polarized BBMEC and Caco-2 monolayers can be affected by P85 unimer inhibition of the P-gp efflux system. The P-gp transporter is expressed at the apical (AP) side of the BBMEC and Caco-2 monolayers and is not expressed at the basolateral (BL) side (16,17). Therefore, we evaluated the R123 transport in both AP to BL and BL to AP directions. This study used 22 μ M P85, to ensure that the copolymer concentration is below the CMC (\approx 67 μ M), i.e. all copolymer is in the form of the unimers (10). Figure 1 summarizes the results of the study with the Caco-2 (panel A) and BBMEC (panel B) monolayers.

In both cell monolayers there is a significant difference in the directionality of the transport of the probe in the assay buffer: the AP to BL flux is much less than the BL to AP flux. This result is consistent with the AP localization of the P-gp efflux system in these cells. The difference in BL to AP vs. AP to BL fluxes is 7.8 times in the case of Caco-2 and 2.3 times in the case of BBMEC cells. More significant directionality effects with the Caco-2 monolayers may be explained by possibly higher P-gp expression or activity in these cells.

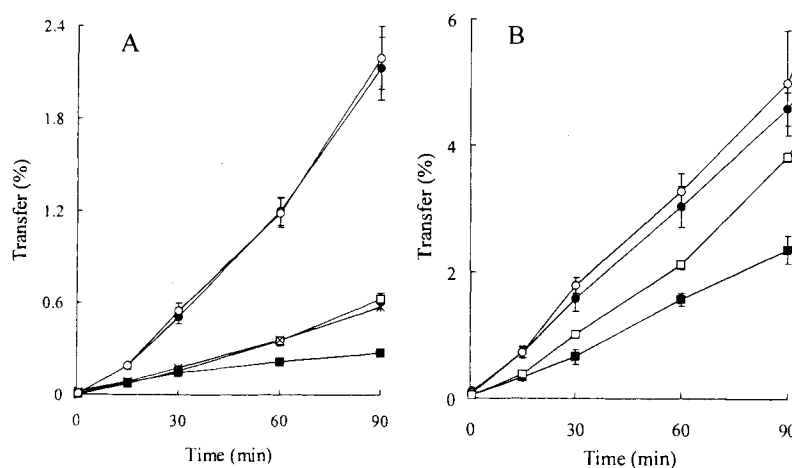


Fig. 1. Flux of R123 in Caco-2 (panel A) and BBMEC (panel B) monolayers. Transport of the probe in AP to BL direction was examined using assay buffer (filled squares) and 22 μ M P85 at the AP side of the cell monolayers (empty squares). Transport of the probe in BL to AP direction was examined using assay buffer (filled circles), 22 μ M P85 at the BL side (empty circles), and 22 μ M P85 at the AP side of the cell monolayers (crosses). Values represent the mean \pm SEM of three monolayers per the treatment group.

Another factor that may contribute to the quantitative differences with those cell models is the effects of paracellular transport of the probe. The P-gp efflux system controls the transcellular component of the drug transport and does not affect the paracellular transport component in these cells. In the BBMEC monolayers the transport of a paracellular marker, mannitol, is ca. 2 times higher than that in the Caco-2 monolayers (data not shown). This suggests that BBMEC monolayers are more "leaky". Higher paracellular permeability may mask the effects of P-gp efflux system in the case of the BBMEC monolayers. As a result, the P-gp controlled directionality difference appears to be greater in the Caco-2 monolayers.

When P85 unimers are administered at the AP side of the cell monolayers there is a significant increase in the AP to BL flux of the probe with both cell models (Fig. 1A,B). The effects of the copolymer unimers are time-dependent. The probe transport increases are observed after 15 min and continue to elevate up to 90 min, which is the end point of this experiment (Fig. 1A,B). This time dependency is consistent with our previous report that approximately 15-min exposures to the P85 unimers are needed to block the P-gp efflux system in the cells (10). Within the 90-min time interval the copolymer unimers did not affect the mannitol flux in both cell monolayers (data not shown). This suggests that the effects of P85 unimers on the R123 flux do not involve paracellular permeability changes. Further, there is no change in the probe flux in BL to AP direction when P85 unimers are added to the BL side of the monolayers (Fig. 1A,B). Therefore, the effects of the P85 unimers are direction-dependent and are observed only when the copolymer is added at the same side of the monolayers where the P-gp efflux pump is expressed. These data convincingly demonstrate that P85 unimers increase transcellular flux of R123 in AP to BL direction in Caco-2 and BBMEC monolayers without affecting the paracellular permeability and this effect appears to be attributable to inhibition of P-gp.

To further demonstrate the specificity of the unimer effect with respect to the P-gp we evaluated whether the inhibition

of the efflux system by the unimers at the AP side affects the directionality of the probe transport. The Caco-2 monolayers were used as a model in this study, because, these cells reveal higher directionality effects. The P85 unimers were added to the AP side of the monolayers to inhibit the P-gp efflux system. The AP to BL and BL to AP fluxes of the probe were characterized. Compared to AP to BL flux in the assay buffer the AP to BL flux with P85 unimers is increased as discussed before. Compared to BL to AP flux in the assay buffer the BL to AP flux with P85 unimers is significantly decreased. It is noteworthy that the fluxes of the probe in both directions become equal when the unimers are present at the AP side (Fig. 1A). In other words the blockade of the P-gp efflux system by the P85 unimers eliminates the transport directionality differences. This result provides additional support for the P-gp mediated effects of the P85 unimers.

Effects of P85 Micelles on R123 Transport

The effects of P85 micelles on cellular accumulation of R123 solubilized in BBMEC and Caco-2 monolayers were previously reported (10,11). These studies demonstrate that the effects of the micelles are very different from those of the P85 unimers. In the case of the micelles enhanced R123 accumulation is either transient (BBMEC monolayers) or saturated (Caco-2 monolayers)—a marked difference with the monotonous increase of the probe uptake with the unimers. These differences are indicative of the multiple mechanisms of the drug transport with the P85 micelles and unimers (10,11). To further validate the hypothesis about the multiple transport mechanisms the present work characterizes the effects of the P85 micelles on the R123 flux in BBMEC and Caco-2 monolayers. The concentrations of the block copolymer chosen for this study were above the CMC to ensure that the micelles were present. With the Caco-2 monolayers we used 11 mM P85. At this concentration over 95% of the probe is incorporated in the P85 micelles (10). Since 11 mM P85 affected the conflu-

ency of the BBMEC monolayers (as determined by increased mannitol permeability) the copolymer concentration was decreased to 2.2 mM in the studies performed on these cells. This concentration of P85 is still above CMC and ca. 50% of the R123 is incorporated in the P85 micelles (10).

The results of the transport studies with the micelles were qualitatively similar in both cell models. As it is shown in Fig. 2A, the co-administration of P85 micelles with R123 decreases the flux of the probe in Caco-2 monolayers in both directions by ca. 3 times. Very similar result was obtained using BBMEC monolayers, although in this case the flux decreases were less significant (ca. 1.8 times), which is probably explained by higher paracellular leakage of the probe with these cells (Fig. 2B). Since the effect of P85 micelle was direction-independent (Fig. 2A) it appears that it has no relevance to the effects on P-gp efflux system. This reinforces the conclusion that the effects of micelles involve different transport mechanisms compared to the unimer effects.

It was previously suggested that the drug administered to the BBMEC monolayers with the micelles is first absorbed by the cell by vesicular transport mechanism and then recycled out of the cell (10). In this paper we present further evidence of the recycling of R123 with the micelles in the BBMEC monolayers. To characterize the dynamics of the R123 interaction with the cells the fluorescence microscopy experiments were performed at various times of exposure of the cell monolayers to the probe (Fig. 3). At the early time point of 5 min R123 fluorescence is localized primarily in the vesicles (Fig. 3A). At 15 min R123 accumulation in cells reaches maximal levels and the probe is spread throughout the cells (Fig. 3B). At 30 min cellular R123 is significantly decreased (Fig. 3C), and at 90 min the probe is cleared from the cells (Fig. 3D). In contrast there was a monotonous increase in probe accumulation in the control monolayers treated by R123 in the assay buffer (Fig. 3E, F).

Further evidence that the micelles enhance the clearance of the drug from the cell monolayers was obtained in the efflux

studies. In these experiments the cell monolayers were pre-loaded with R123 in assay buffer and then the drug efflux to either AP or BL side was determined in the presence and absence of P85 micelles. The study was performed both on BBMEC and Caco-2 monolayers with the same result: the micelles increased the efflux of the probe by 2 to 3 times at the same side where they were added (Table 1). This suggests that the enhanced efflux of R123 may be a reason for the decreased permeability of this probe in the presence of the micelles.

R123 Transport in Ins-Vectorized Micelles

In the final part of this work we evaluated the effect of the ligand conjugated to the P85 micelles on the transport of the solubilized drug in the cell monolayers. The model chosen for this study characterized the effects of insulin (Ins) as a vector for modulating the receptor-mediated transport of the micelles in the BBMEC monolayers. The rationale for these studies is the previous report by Miller et al. supporting insulin transport through BBMEC monolayers (transcytosis) in AP to BL direction (18). In the first experiment, R123 was administered at the AP side of the cell monolayers with P85 micelles (2.2 mM P85) containing varying concentration of the Ins-P85 conjugates (1 to 100 nM). This experiment determined the optimal concentration of Ins-P85 conjugate (1 nM) that does not affect the permeability of the cell monolayers with respect to mannitol. Figure 4 shows the effect of the vectorized micelle formulation on the AP to BL transport of R123 in the BBMEC monolayers. In the presence of the Ins vector the transport of the probe was increased by ca. 2 to 3-times compared to the transport of the probe in the Ins-free micelles or in the micelles with the same concentration of the unconjugated Ins. The transport of the probe in Ins-modified micelles significantly exceeded that in the control assay buffer group. It is noteworthy that Ins-P85 conjugate did not affect the transport of the probe

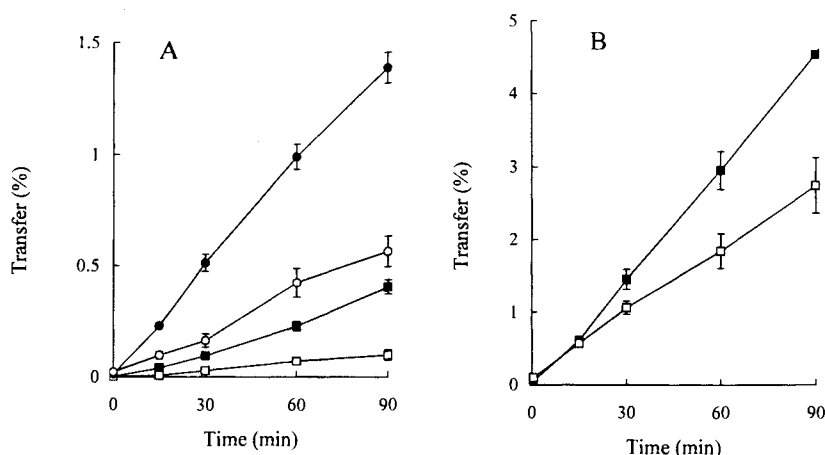


Fig. 2. Flux of R123 in Caco-2 (panel A) and BBMEC (panel B) monolayers. Transport of the probe in AP to BL direction in Caco-2 and BBMEC monolayers was examined using assay buffer (filled squares) and 2.2 mM P85 (BBMEC) or 11 mM P85 (Caco-2) at the AP side of the cell monolayers (empty squares). Transport of the probe in BL to AP direction in Caco-2 monolayers was examined using assay buffer (filled circles) and 11 mM P85 at the BL side (empty circles). Values represent the mean \pm SEM of three monolayers per the treatment group.

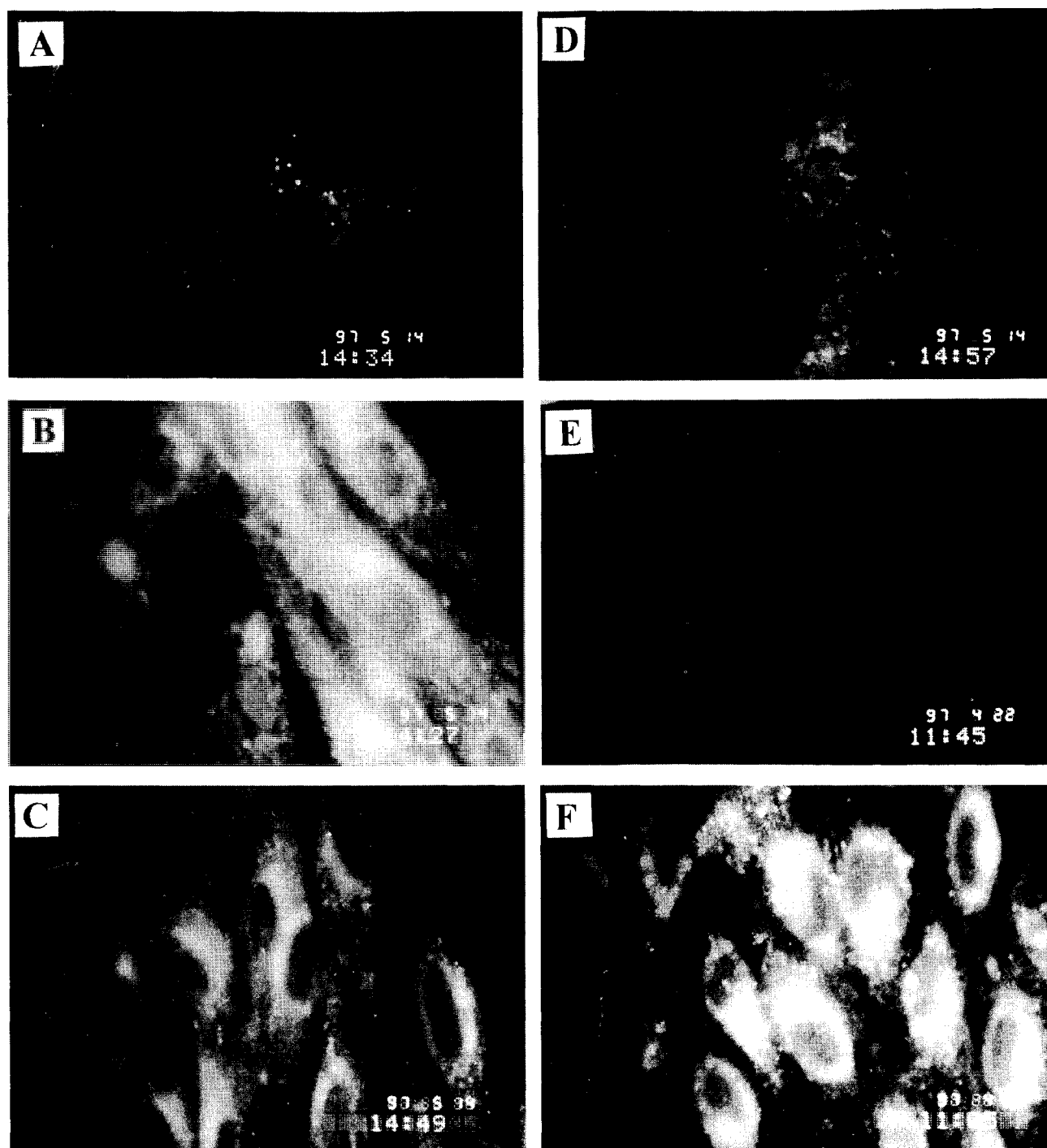


Fig. 3. Fluorescence microscopy of R123 accumulation in BBMEC monolayers following various times of exposure with 2.2 mM P85: (A) 5 min, (B) 15 min, (C) 30 min, and (D) 90 min. R123 accumulation in assay buffer is shown for comparison: (E) 15 min, and (F) 90 min.

with the P85 micelles in the Caco-2 cell monolayers that do not have Ins receptor at the AP side (data not presented).

To validate the receptor-mediated mechanism of the probe transport in the Ins-vectorized micelles the competitive inhibition of the transport with an excess of free Ins was studied at the 90-min time point (Table 2). The results of this experiment suggests that in the presence of the 17 nm free Ins the transport of the probe in Ins-vectorized micelles is decreased down to

the level observed with the Ins-free micelles. Therefore, conjugation of the micelles with Ins ligand results in a receptor-mediated redirection of the drug transport from AP to BL side of the cell monolayers.

DISCUSSION

The proposed mechanisms of drug transport in the polarized cell monolayers using Pluronic block copolymers basing

Table 1. Efflux of R123 in BBMEC and Caco-2 Monolayers

Cell model	Time, min	Efflux of R123, % of loading solution ^a			
		AP side		BL side	
		assay buffer	micelles	assay buffer	micelles
BBMEC	0.5	1.16 ± 0.12	1.39 ± 0.44	1.27 ± 0.15	1.72 ± 0.15
	9	2.32 ± 0.28	2.72 ± 0.71	2.04 ± 0.21	3.99 ± 0.51
	15	2.70 ± 0.35	3.41 ± 0.83	2.33 ± 0.21	4.62 ± 0.60
	22	2.93 ± 0.36	4.19 ± 0.81	2.62 ± 0.22	5.05 ± 0.66
Caco-2	0.5	0.07 ± 0.01	0.28 ± 0.04	0.12 ± 0.02	0.35 ± 0.02
	9	0.22 ± 0.01	0.67 ± 0.09	0.37 ± 0.02	1.02 ± 0.10
	15	0.25 ± 0.01	0.86 ± 0.09	0.48 ± 0.01	1.23 ± 0.01
	22	0.30 ± 0.02	0.96 ± 0.11	0.54 ± 0.02	1.37 ± 0.13

^a Values represent the mean ± SEM of three monolayers per the treatment group.

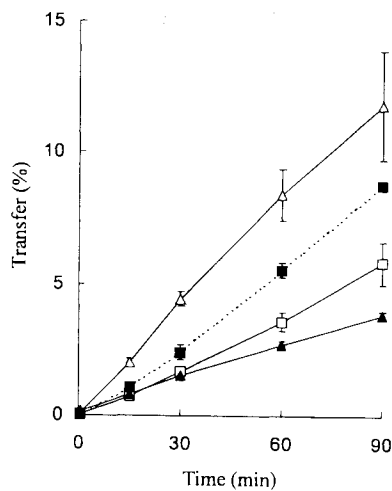


Fig. 4. Flux of R123 in BBMEC monolayers. Transport of the probe in AP to BL direction was examined using assay buffer (filled squares, dotted line), 2.2 mM P85 (empty squares), 2.2 mM P85 with 1 nM Ins free Ins (filled triangles) and 2.2 mM P85 with 1 nM Ins-P85 conjugate (empty triangles) at the AP side of the cell monolayers. Values represent the mean ± SEM of three monolayers per the treatment group.

Table 2. Effects of Ins on the Flux of R123 Solubilized in P85 Micelles in AP to BL Direction in BBMEC Monolayers

System studied	Transfer at 90 min, % ^a
2.2 mM P85	1.94 ± 0.14
2.2 mM P85 with 1 nM Ins-P85 conjugate	3.85 ± 0.68
2.2 mM P85 with 1 nM Ins-P85 conjugate and 17 nM of free Ins ^b	2.28 ± 0.28

^a Values represent the mean ± SEM of three monolayers per the treatment group.

^b Concentration of the free Ins for this experiment was determined based on the data on competitive inhibition of Ins binding and internalization in BBMEC monolayers reported by Miller *et al.* (18).

on the results of this work are schematically presented in Fig. 5. The unimer concentration of the block copolymer inhibits P-gp efflux system expressed at the AP side of the BBMEC and Caco-2 cell monolayers. This results in the increase in the drug permeability in AP to BL direction (Fig. 5A). With high concentrations of the block copolymer significant portions of the drug become incorporated into the micelles. At physiological conditions P85 micelles represent aggregates of ca. 60 block copolymer molecules with a hydrodynamic diameter of ca. 15 nm (19). The hydrophobic core of such micelles hosting the drug molecules consists of PO chain segments and their hydrophilic shell is made of EO chains, i.e. the same material that is used in the hydrophilic corona of many Stealth™ liposomes (20). The micelle-solubilized drug enters the cell via the fluid-phase endocytosis (Fig. 5B). The vesicular transport mechanism appears to be the most likely for the EO covered particles with the size of the P85 micelles. Indeed it is supported both by the previously reported energy-dependency studies in the BBMEC monolayers (10) and fluorescence microscopy studies in this

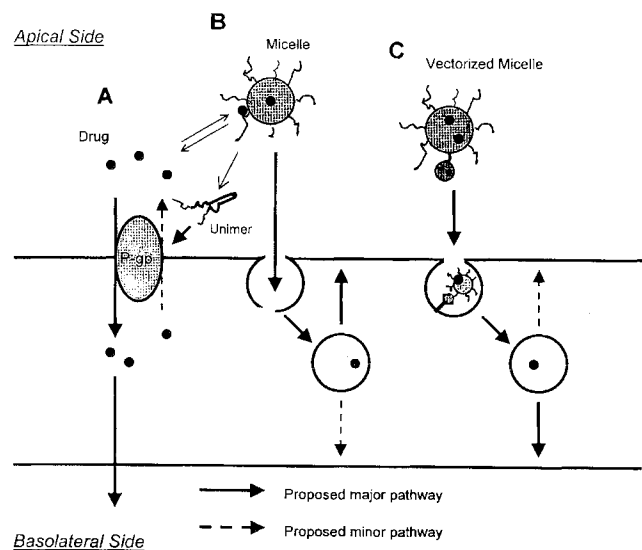


Fig. 5. Proposed mechanism of drug transport in BBMEC and Caco-2 cell monolayers with Pluronic block copolymers: (A) inhibition of P-gp, (B) fluid phase endocytosis, and (C) adsorptive endocytosis (BBMEC only).

work suggesting vesicular localization of the probe in the cells after 5 min exposures. In the absence of the specific vector capable of affecting micelle processing in the cell via the receptor-mediated pathway the drug is then recycled out of the cells (Fig. 5B). While the mechanism of the drug recycling in the cells is presently unknown it appears to involve enhanced efflux of the probe induced by the micelles. The efflux is directed to the same side where the micelles are administered, which results in the decrease of the drug transport across the cell monolayers (Fig. 5B).

It is noteworthy that the transport effects observed with the micelles are independent on the side of administration to the monolayers while the effects of the unimers are strongly polarized and are associated with the AP localization of the P-gp efflux system. Very similar transport phenomena were recently described for the drug transport with the nonionic detergents, such as Cremphor EL and Tween 60 using Caco-2 cell monolayers (21,22). In those work the inhibition of P-gp efflux system with the detergent was supported by the studies of the model drug transport in the AP to BL direction. Because various drugs are transported in a manner similar to R123 (21,22,24–26) our results suggest the Pluronic copolymers may be useful in enhancing the absorption of such molecules across the barriers of the gut and central nervous system. These copolymers have been previously characterized as much more potent inhibitors of the P-gp efflux system and hypersensitizing agents in multidrug resistant cell lines compared to the nonionic detergents (8–11). The effects of the Pluronic block copolymers probably involve interactions of their unimers with the cell membranes resulting in the nonspecific changes in membrane properties such as ion transport, potential, and, possibility, fluidity (8). These interactions appear to affect P-gp efflux pump and may also involve changes in various membrane protein functions. There is at least one indication in the literature, that P85 block copolymer can inhibit the efflux of ATP outside of the cell (23). Furthermore, we have recently demonstrated that the P85 unimers inhibit the organic anion transporter, MRP, in several cells including BBMEC (paper in preparation). This provides preliminary evidence that Pluronic block copolymers can be used to increase membrane transport of very broad drug classes, characterized by decreased permeability in cancer and normal tissues due to the effects of the membrane transporters. The effects of the Pluronic block copolymer unimers were shown to strongly depend on the copolymer hydrophobic-hydrophilic balance (11), which potentially provides the possibility to optimize formulations for improved transport of drugs. A specific implication of this work is in the use of Pluronic block copolymers to increase brain and oral absorption of select drugs.

The effects of the Pluronic micelles appear to be similar to those reported for the micelles of the nonionic detergents (22): that is the micelles decrease the drug flux in the cell monolayers in either direction. While we disagree with Nerurkar et al. (22) about the mechanisms of the micelle effects and demonstrate here that the micelle-incorporated drug in fact enters the cell and then recycles back, we believe that the micelle-mediated transport phenomena observed in this and earlier work (10,11,22) are important. The significance of these observations is even better understood in the context of the recycling of the drug with the micelles. Indeed many drugs are so hydrophobic that micellar solubilization (including incorporation in the block copolymer micelles (1–6)) appears to be

one of the few methods for the drug administration. Now we demonstrate that not only the release of the drug can be decelerated under these conditions, but also the drug itself can either be prevented from the transport into the cell or “washed out” of the cells. It is therefore even more important, that the direction of transport of the micelle-incorporated drug can be modified by conjugating the micelles with the ligand capable of adsorptive endocytosis in the cell. By demonstrating that Ins-vectorized Pluronic block copolymer micelles can be redirected in the BBMEC monolayers from AP to BL side (Fig. 5C) we suggest that this mechanism can be of significance for enhancing brain transport of the micelle-solubilized drugs. This links the current study and our earlier paper (2), which is the first report on using block copolymer micelles as microcontainers for drug delivery, in particular the Ins-conjugated P85 micelles for the *in vivo* delivery of the solubilized neuroleptic to the brain.

ACKNOWLEDGMENTS

We would like to acknowledge the support of this work by the National Institutes of Health (R01 NS36229-01A1) and Nebraska Research Initiative. We would like to thank Dr. R. T. Borchardt for providing Caco-2 cells and BASF Co. for the gift of P85. We also would like to thank Dr. Valery Alakhov for the discussion of the results of this paper.

REFERENCES

1. S. Y. Lin and Y. Kawashima. Pluronic surfactants affecting diazepam solubility, compatibility, and adsorption from i.v. admixture solutions. *J. Parenter. Sci. Technol.* **41**:83–87 (1987).
2. A. V. Kabanov, V. P. Chekhonin, V. Yu. Alakhov, E. V. Batrakova, A. S. Lebedev, N. S. Melik-Nubarov, S. A. Arzhakov, A. V. Levashov, G. V. Morozov, E. S. Severin, and V. A. Kabanov. The neuroleptic activity of haloperidol increases after its solubilization in surfactant micelles. Micelles as microcontainers for drug targeting. *FEBS Lett.* **258**:343–345 (1989).
3. A. Rolland, J. O'Mullane, P. Goddard, L. Brookman, and K. Petrak. New macromolecular carriers for drugs. 1. Preparation and characterization of poly(oxyethylene-b-isoprene-b-oxyethylene) block copolymer aggregates. *J. Appl. Polym. Sci.* **44**:1195–1203 (1992).
4. G. S. Kwon, M. Naito, M. Yokoyama, T. Okano, Y. Sakurai, and K. Kataoka. Physical entrapment of adriamycin in AB block copolymer micelles. *Pharm. Res.* **12**:192–195 (1995).
5. S. A. Hagan, A. G. A. Coombes, M. C. Garnett, S. E. Dunn, M. C. Davies, L. Illum, S. S. Davis, S. E. Harding, S. Purkiss, and P. R. Gellert. Polylactide-poly(ethylene glycol) copolymers as drug delivery systems. I. Characterization of water dispersible micelle-forming systems. *Langmuir* **12**:2153–2161 (1996).
6. X. Zhang, J. K. Jackson, and H. M. Burt. Development of amphiphilic block copolymers as micellar carriers of taxol. *Int. J. Pharm.* **132**:195–206 (1996).
7. A. V. Kabanov, E. V. Batrakova, N. S. Melik-Nubarov, N. A. Fedoseev, T. Yu. Dorodnich, V. Yu. Alakhov, V. P. Chekhonin, I. R. Nazarova, and V. A. Kabanov. A new class of drug carriers: micelles of poly(oxyethylene)-poly(oxypropylene) block copolymers as microcontainers for drug targeting from blood in brain. *J. Contr. Rel.* **22**:141–158 (1992).
8. V. Yu. Alakhov, E. Yu. Moskaleva, E. V. Batrakova, and A. V. Kabanov. Hypersensitization of multidrug resistant human ovarian carcinoma cells by Pluronic P85 block copolymer. *Bioconjugate Chem.* **7**:209–216 (1996).
9. A. Venne, S. Li, R. Mandeville, A. V. Kabanov, and V. Yu. Alakhov. Hypersensitizing effect of Pluronic L61 on cytotoxic

- activity, transport, and subcellular distribution of doxorubicin in multiple drug-resistant cells. *Cancer Res.* **56**:3626–3629 (1996).
10. D. W. Miller, E. V. Batrakova, T. O. Waltner, V. Yu. Alakhov, and A. V. Kabanov. Interactions of Pluronic block copolymers with brain microvessel endothelial cells: evidence for multiple absorption pathways. *Bioconjugate Chem.* **8**:649–657 (1997).
 11. E. V. Batrakova, H.-Y. Han, V. Yu. Alakhov, D. W. Miller, and A. V. Kabanov. Effects of Pluronic block copolymers on drug absorption in Caco-2 cell monolayers. *Pharm. Res.* **15**:850–855 (1998).
 12. D. W. Miller, K. L. Audus, and R. T. Borchardt. Application of cultured bovine brain endothelial cells of the brain microvasculature in the study of the blood-brain barrier. *J. Tiss. Clt. Meth.* **14**:217–224 (1992).
 13. J. Fogh, J. M. Fogh, and T. J. Orfeo. One hundred and twenty seven cultured human cell lines producing tumors in nude mice. *J. Natl. Cancer Inst.* **59**:221–226 (1977).
 14. P. Artursson. Epithelial transport of drugs in cell culture. I: A model for studying the passive diffusion of drugs over intestinal absorptive (Caco-2) cells. *J. Pharm. Sci.* **79**:476–482 (1990).
 15. D. C. Dawson. Na and Cl transport across the isolated turtle colon: parallel pathways for transmural ion movement. *J Membrane Biol.* **37**:213–233 (1977).
 16. K. I. Hosoya, K. J. Kim, and V. H. Lee. Age-dependent expression of P-glycoprotein gp170 in Caco-2 cell monolayers. *Pharm. Res.* **13**:885–890 (1996).
 17. E. Beaulieu, M. Demeule, L. Ghitescu, and R. Beliveau. P-glycoprotein is strongly expressed in the luminal membranes of the endothelium of blood vessels in the brain. *Biochem. J.* **326**:539–544 (1997).
 18. D. W. Miller, B. T. Keller, and R. T. Borchardt. Identification and distribution of insulin receptors on cultured bovine brain microvessel endothelial cells: possible function in insulin processing in the blood-brain barrier. *J. Cell. Physiol.* **161**:333–341 (1994).
 19. A. V. Kabanov, I. R. Nazarova, I. V. Astafieva, E. V. Batrakova, V. Yu. Alakhov, A. A. Yaroslavov, and V. A. Kabanov. Micelle formation and solubilization of fluorescent probes in poly(oxyethylene-b-oxypropylene-b-oxyethylene) solutions. *Macromolecules* **28**:2303–2314 (1995).
 20. D. Lasic and F. Martin, Eds. *Stealth Liposomes*. CRS Press, Boca Raton, 1995.
 21. M. M. Nerurkar, P. S. Burton, and R. T. Borchardt. The use of surfactants to enhance the permeability of peptides through Caco-2 cells by inhibition of an apically polarized efflux system. *Pharm. Res.* **13**:528–534 (1996).
 22. M. M. Nerurkar, N. F. H. Ho, P. S. Burton, T. J. Vidmar, and R. T. Borchardt. Mechanistic roles of neutral surfactants on concurrent polarized and passive membrane transport of a model peptide in Caco-2 cells. *J. Pharm. Sci.* **7**:813–821 (1997).
 23. V. I. Slepnev, L. E. Kuznetsova, A. N. Gubin, E. V. Batrakova, V. Yu. Alakhov, and A. V. Kabanov. Micelles of poly(oxyethylene)-poly(oxypropylene) block copolymer (pluronic) as a tool for low-molecular compound delivery into a cell. Phosphorylation of intracellular proteins with micelle incorporated [γ -³²P]ATP. *Biochem. Internat.* **26**:587–595 (1992).
 24. D. Leveque, and F. Jehl. P-glycoprotein and pharmacokinetics. *Anticancer Res.* **15**:331–336 (1995).
 25. C. G. Lee, M. M. Gottesman, C. O. Cardarelli, M. Ramachandra, K. T. Jeang, S. V. Ambudkar, I. Pastan, and S. Dey. HIV-1 protease inhibitors are substrates for the MDR1 multidrug transporter. *Biochemistry* **37**:3594–3601 (1998).
 26. N. Drion, P. Risede, N. Cholet, C. Chanez, J. M. Scherermann. Role of P-170 glycoprotein in colchicine brain uptake. *J. Neurosci. Res.* **49**:80–88 (1997).