Dodecylphosphocholine-Mediated Enhancement of Paracellular Permeability and Cytotoxicity in Caco-2 Cell Monolayers

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Abstract □ The intestinal epithelium is a significant barrier for oral absorption of hydrophilic drugs because they cannot easily traverse the lipid bilayer of the cell membrane and their passage through the intercellular space (paracellular transport) is restricted by the tight junctions. In this report we show that dodecylphosphocholine (DPC) can improve the paracellular permeability of hydrophilic compounds across Caco-2 cell monolayers by modulating the tight junctions. The results show that the alkyl chain as well as the zwitterionic head group of DPC are required for its activity. DPC appears to act by modulating the permeability of tight junctions as evidenced by the fact that treatment of Caco-2 cell monolayers by this agent results in a decreased transepithelial electrical resistance (TEER), increased permeability of paracellular markers (e.g., mannitol) with no change in the permeability of the transcellular marker testosterone, and redistribution of the tight junction-associated protein ZO-1. The effect of DPC on Caco-2 cells (e.g., decrease in TEER) is reversible, and is not caused by gross cytotoxicity (as indicated by the MTT test) or by nonspecific disruption of the cell membrane (as indicated by only slight nuclear staining due to the nonpermeable DNA-specific dye propidium iodide). We propose in the present study a parameter, potency index, that allows comparison of various enhancers of paracellular transport in relation to their cytotoxicity. The potency index is a ratio between the IC_{50} value (concentration at which 50% inhibition of control mitochondrial dehydrogenase activity occurs in the MTT test) and the EC_{50} value (concentration at which TEER drops to 50% of its control (untreated) value). By this parameter, DPC is significantly safer than the commonly used absorption enhancer palmitoyl carnitine (PC), which has the potency index of ~1 (i.e., no separation between effective and toxic concentration).

Introduction

The intestinal epithelium represents a major barrier to absorption of orally administered drugs and nutrients into systemic circulation. The translocation of drug molecules across the intestinal epithelium occurs by passive diffusion via transcellular or paracellular route, or through carrier-mediated active or facilitated transport. The intercellular space of the intestinal epithelium restricts the passage of molecules because of the presence of junctional complexes (tight junctions, intermediate junctions, and desmosomes).[1,2] Hence, the intestinal epithelium is a significant barrier for hydrophilic molecules because they cannot easily traverse the lipid bilayer of the cell membrane, and their passage through the intercellular space is restricted by the tight junctions, i.e., zonula occludens (ZO).

Numerous classes of compounds with diverse chemical properties, including detergents, surfactants, bile salts, Ca^{2+} chelating agents, fatty acids, phospholipids etc., have been reported to enhance the intestinal absorption of not only small drug molecules but also relatively large polypeptides drugs.[3–19] Many of these enhancers act as detergents/surfactants and increase the transcellular transport of drugs by disrupting the structure of the lipid bilayer and rendering the cell membrane more permeable and/or by increasing the solubility of insoluble drugs. Others act as Ca^{2+} chelators, and improve the paracellular transport of hydrophilic drug molecules by disrupting the tight junctions after the removal of extracellular Ca^{2+} from the medium. Several phospholipid-like agents, many of which are lysophosphatidylcholines,[8–12] medium chain fatty acids,[16–18] and acyl carnitines (e.g., palmitoyl carnitine),[3,5,13–15] appear to increase the paracellular permeability of hydrophilic compounds by mechanism(s) other than Ca^{2+} chelation. Although the exact mechanism(s) of action for many of these agents is yet to be determined, at least one mechanism by which they appear to exert their effect on the tight junctions is via upregulation of intracellular Ca^{2+}.[16–18]

A major concern regarding the use of these agents to enhance intestinal absorption is their postulated linkage to the epithelial damage and toxicity associated with the compromised mucosal function and morphology.[6,7] While these compounds are extensively used as absorption/transport enhancers, it is difficult to assess their relative potency and toxicity because of limited data available on concentration vs effect and also because of the use of different agents in different model systems. For example, palmitoyl carnitine (PC) has been used as a transport enhancer at a concentration range of 0.1–0.4 mM[14,15] in Caco-2 cell culture model. In our hands, PC was effective as transport enhancer in Caco-2 cell culture model at concentrations above 0.4 mM (cf., Figures 2, 5).

Our preliminary results have shown that dodecylphosphocholine (DPC), which lacks the glycerol moiety present in lysophoshatidylcholines, enhances the transport of hydrophilic compounds across Caco-2 cell monolayers by modulating the tight junctions (and perhaps other junctional complexes).[20] These results suggest that the activity of LGPC and other lysophosphatidylcholines[9] as enhancers of paracellular permeability is due to the presence of alkyl chain and a zwitterionic group and not due to the glycerol functionality. DPC contains these structural features in the simplest possible configuration. Hence, in this paper we report a systematic evaluation of DPC as an enhancer of paracellular transport of hydrophilic compounds across Caco-2 cell monolayers. Furthermore, we have compared DPC with several absorption enhancers in order to determine its potency as a paracellular transport enhancer and its cytotoxicity relative to the potency and cytotoxicity of other well-established absorption enhancers. While several

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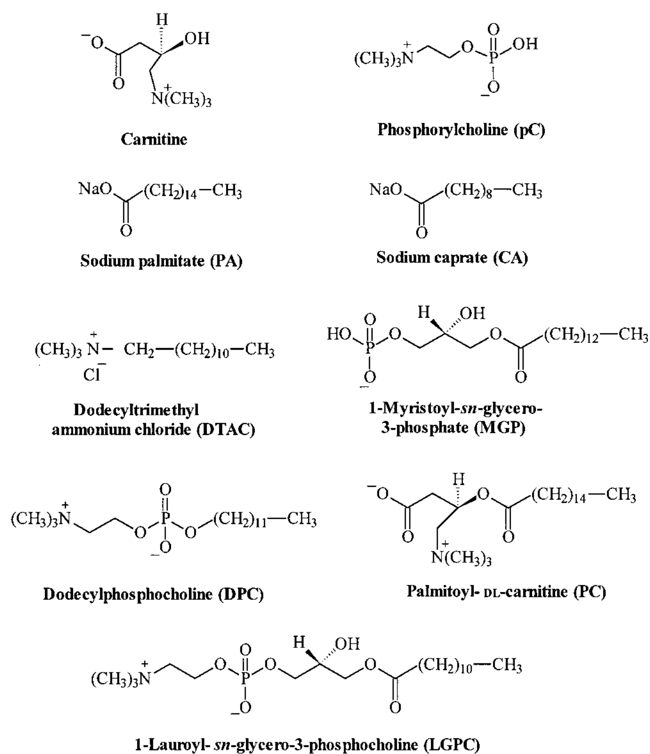

Figure 1—Chemical structures of the compounds evaluated as transport/absorption modulators.

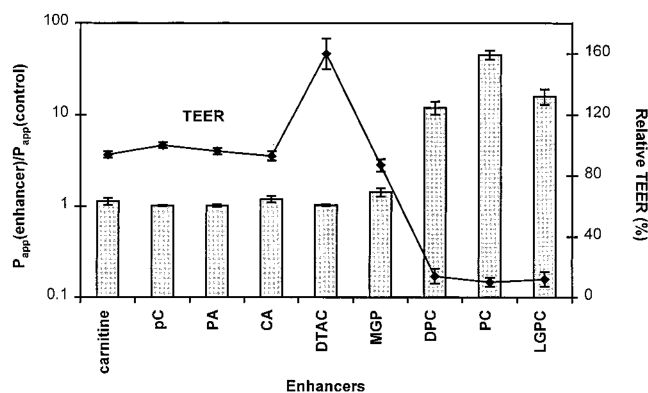

Figure 2—Effect of absorption enhancers and their analogues on TEER across Caco-2 cell monolayers. All the agents (0.75 mM) were applied to the apical compartment of the monolayers for 20 min at room temperature. Mannitol transport was measured (P_{app}) after the treatment by the agents and was compared to the control P_{app} value obtained with the untreated cells. (Abbreviations not used in the text: PA, palmitic acid; CA; sodium caprate). ◆ :P_{app}(treated)/P_{app}(control).

reports of the use of Caco-2 cells to evaluate toxicity of absorption enhancers have appeared recently,[20–24] we propose in the present study a parameter, potency index, that allows comparison of various paracellular transport enhancers in relation to their cytotoxicity.

Materials and Methods

Reagents—Caco-2 cells were purchased from American Type Culture Collection (Rockville, MD). Eagle's Minimum Essential Medium (EMEM), 0.25% trypsin/0.02% ethylenediamine–tetraacetic acid–sodium salt (EDTA-4Na), goat serum, and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY). Nonessential amino acids (NEAA), Hank's balanced salt solution (HBSS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT), palmitoyl-DL-carnitine chloride (PC), sodium caprate (CA), sodium dodecyl sulfate (SDS), palmitic acid (PA), dodecyltrimethylammonium chloride (DTAC), phosphorylcholine (pC), and DL-carnitine hydrochloride were purchased from either Sigma (St. Louis, MO) or Aldrich (Milwaukee, WI). Dodecylphosphocholine (DPC), 1-myristoyl-*sn*-glycero-3-phosphate sodium (MGP), 1-lauroyl-*sn*-glycero-3-phosphocholine (LGPC) were purchased from Avanti Polar Lipids (Alabaster, AL). *N*-(2-Hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid (HEPES) and penicillin /streptomycin were obtained through the Tissue Culture Facility (UNC at Chapel Hill, NC). Transwell plates and inserts (12 wells/plate, 3.0 μm pore and 1.0 cm^2 area, polycarbonate) were purchased from Corning-Costar (Cambridge, MA). [^{3}H]Mannitol, [^{3}H]-PEG-900, [^{3}H]-PEG-3500, and [^{14}C]testosterone were obtained from DuPont NEN (Boston, MA). Lucifer Yellow (LY) and propidium iodide were obtained from Molecular Probes (Eugene, OR).

Antibodies—Rabbit polyclonal antibody against ZO-1 was purchased from Zymed Laboratory, Inc. (San Francisco, CA). Fluorescein isothiocyanate (FTIC)-labeled Goat anti-Rabbit IgG antibodies were purchased from Sigma (St. Louis, MO).

Caco-2 Cell Culture—Caco-2 cells were maintained in EMEM, containing 10% fetal bovine serum, 1% l-glutamine, 1% NEAA, and antibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin) in 75 cm^2 culture flasks. The cultures were kept at 37 °C in an atmosphere of 5% CO_2, 95% air, and 90% relative humidity. Cells were passaged after 95% confluency and were seeded with a density of 1.2×10^5 cells/mL onto porous polycarbonate filter membranes with a pore size of 3.0 μm and a surface area of 1.0 cm^2. Cells of passage number 40–55 were used throughout. Media were changed every 2 days after seeding until late confluence (21–23 days). Just before the experiments, the culture medium was replaced with transport buffer (HBSS contained 25 mM HEPES and glucose) and incubated for 1 h at 37 °C.

Preparation of Enhancer Solution—All the compounds being evaluated as transport enhancers were dissolved in a HBSS that contained 25 mM HEPES at pH 7.4. The stock solutions (50 mM) of the test compounds were obtained by dissolving them in HBSS-buffer/ethanol (60:40, v/v) and were stored at −20 °C. Just before the experiments, the enhancer solutions were thawed and diluted to the appropriate concentrations by HBSS buffer and sonicated for 5 min in an ice bath.

Critical Micelle Concentration (CMC)—The CMC of all tested enhancers was determined by measurement of surface tension as a function of concentration of enhancers in HBSS (contained 25 mM glucose and 25 mM HEPES) buffer at room temperature. For this study, a Kruss K10T digital tensionmeter (Hamburg, Germany) was used.

Cell Viability Assay—The cell viability was measured by the MTT test[25] as performed for evaluation of the cytotoxic effects of absorption enhancers to Caco-2 cells.[26] Approximately 3×10^3 cells (in 100 μL of cell culture medium) were seeded into the wells in a 96-well tissue culture plate (Corning-Costar, Cambridge, MA). The cells were then cultured under the same condition as the monolayer preparation for 72–96 h before use. Just prior to the start of each experiment, the medium was removed from the wells, and 100 μL of the enhancer solution in HBSS was added to each well. After exactly 20 min, 20 μL of a 5 mg/mL MTT solution was added to each well, and the cells were incubated for another 90 min. Then 100 μL of 5% SDS in 0.02 M HCl/isobutanol (1:1, v/v) solution was added to stop the reaction and solubilize the reaction products. The absorption was measured at 590 nm with a multiwell scanning spectrophotometer (Bio-Rad, Hercules, CA).

Transepithelial Electrical Resistance (TEER)—The monolayers were treated on the apical side with the absorption enhancers at various concentrations dissolved in 0.5 mL of HBSS. The volume of HBSS on the basolateral side was 1.5 mL. Measurements of the effects of these enhancers on TEER across Caco-2 monolayers were performed using an EVOM Epithelial Tissue Voltohmmeter (World Precision Instruments, Sarasota, FL) at room temperature and 37 °C. The resistance due to the cell monolayers was determined in the presence or absence of the blank filter and the HBSS. The control TEER values were in the range of 500–600 $\Omega \cdot cm^2$.

Cytoplasmic Membrane Permeability—The DNA-intercalating dye, propidium iodide, was used to discern cells with damaged membranes. Caco-2 cells (5.0×10^4 cells/mL) were seeded on to glass coverslips. After 5 days, the completely confluent cell monolayers were used for propidium iodide staining. After expo-

sure by DPC at 0.75 mM, 1.0 mM, and 1.25 mM, the monolayers were rinsed twice with PBS and then incubated with propidium iodide (30 μg/mL PBS) for 3 min. After rinsing twice with PBS, the cells were fixed for 10 min in 3.7% formaldehyde in PBS on ice and then rinsed four more times in PBS. In a separate experiment, the same DPC-exposed monolayer samples, after rinsing with PBS, were incubated in cell culture medium for 8 h and then stained by propidium iodide. Experiments excluding the enhancer were run as controls. The preparations were mounted on glass slides in a 1:1 solution of PBS and glycerol and examined under a fluorescence microscope.

Immunohistochemistry—Caco-2 cell monolayers were prepared as described under "Cell Culture" for transport experiments, and those with TEER values 600−800 Ω·cm^2 were used for the immunohistochemistry study. The cell monolayers were first treated with 0.75 mM DPC for 20 min. Then the monolayer was washed twice after removal of DPC-containing medium. The cells were fixed in 3.7% paraformaldehyde, rinsed in 1x PBS and permeabilized using 0.25% Triton X-100. After further rinsing, the cell monlayer was blocked with PBS containing 10% FCS, 10% goat serum, and 0.2% Tween 20. The primary antibody (rabbit polyclonal antibody against ZO-1), diluted in PBS containing 10% FCS/10% goat serum/0.2% Tween 20, was applied to the cell monolayers and incubated for 2 h. After several washes with 1x PBS/0.2% Tween 20, the monolayer was blocked again and then incubated for another 2 h with the FITC-labeled goat anti-rabbit IgG antibody that was diluted in PBS containing 10% FCS/10% goat serum/0.2% Tween 20. After rinsing in PBS, the filter was cut out, mounted in glass slides, and examined using Zeiss confocal microscope fitted with 60× objective. ZO-1 staining excluding DPC was run as a control. The final images were stored digitally and transferred to Adobe Photoshop for graphical representation.

Transport Experiments—Transport studies were performed at room temperature on filter-grown Caco-2 monolayers with [^{3}H]-labeled mannitol (80 μM, 0.5 μCi/mL), [PEG-900 (100 μM, 0.5 μCi/mL) and PEG-3500 (100 μM, 0.5 μCi/mL), [^{14}C]testosterone (100 μM, 0.1 μCi/mL), or LY (100 μM). The enhancer solution in HBSS (0.5 mL) was added to the apical side of the cells and 1.5 mL of HBSS was added to the basolateral side. After treatment for 20 min, the enhancer solutions were discarded, and the monolayers were washed once with fresh HBSS. Then 0.5 mL of HBSS solution containing one of the compounds was applied to the apical side. Transport rates were determined by measuring the radioactivity/fluorescence associated with the transported compounds present in the BL side at 20-min intervals. The amount of radioactivity was measured by liquid scintillation counting in a PACKARD 1600 TR analyzer (Downers Grove, IL). Transported LY was quantitated with Perkin-Elmer LS50B Luminescence Spectrometer (Beaconsfield, Buckinghamshire, England).

Data Analysis—All transport experiments were carried out under sink conditions, such that the concentration of the compounds in the receiver compartment was always less than 10% of the concentration in the donor compartment. TEER was measured prior to each experiment to ensure the confluency of the cell monolayer. The apparent permeability coefficient was calculated using the following equation:

$$P_{app} = (\mathrm{d}Q/\mathrm{d}t)\cdot(1/A)\cdot(1/C_o)$$

where $\mathrm{d}Q/\mathrm{d}t$ (mol transported/sec) is the flux of the marker compound across Caco-2 cell monolayer, A (cm^2) represents the diffusional area of the inserts, and C_o (M) denotes the initial concentration of marker compound in the donor compartment. All measurements and transports were in triplicate and expressed as mean ± SD values.

Results

Comparative Effect of DPC, PC, and LGPC on TEER and Mannitol Transport Across Caco-2 Cell Monolayers—Because both TEER and mannitol permeability across Caco-2 cells are indicators of tight junction integrity of the cell monolayer, relative effect of compounds on these two indicators reflects their relative ability to modulate tight junctions. PC[15] and lysophosphosphatidylcholines[9] (e.g., LGPC) have been reported as absorption enhancers. The structural features that are common to these very different classes of molecules include a several-carbon-long alkyl chain, an anionic functionality (carboxyl or phosphate group), and a cationic functionality (trimethylaminoalkyl group). We have chosen DPC as a model compound to confirm the role of these structural features in affecting the paracellular permeability. This is because DPC contains all three structural moieties in relatively simple configuration (see Figure 1). In a preliminary study,[20] we have reported that DPC, PC, and LGPC (at 0.75 mM) cause a decrease in TEER to less than 10% of the control value and a significant increase in mannitol permeability across Caco-2 cell monolayers. To elucidate the common structural features that may be responsible for the effect of these compounds on tight junctions, we have evaluated the effect of several other compounds that represent only a partial structure of DPC, PC, or LGPC on TEER and mannitol permeability across Caco-2 cell monolayers at the same concentration (0.75 mM) as that used for the above three compounds (Figures 1 and 2). Interestingly, compounds with an 11- to 15-carbon alkyl chain and either an anionic group or a cationic group (e.g., sodium caprate,[27] palmitate,[27] DTAC, or MGP) did not show any effect on TEER or mannitol transport at the concentration used. Similarly, compounds containing the zwitterionic functionality without the alkyl chains (e.g., carnitine or phosphorylcholine) did not cause any change in TEER or permeability of mannitol. These findings are in agreement with previous findings that the structural components of PC, i.e., carnitine chloride and palmitic acid, had no effect on the transport of paracellular markers across Caco-2 cell monolayers.[15] While DPC, LGPC, and PC appear different structurally, they all contain an alkyl chain and a zwitterionic functionality. All three compounds are quite effective at causing a decrease in TEER and an increase in mannitol permeability across Caco-2 cells. Thus, it is reasonable to conclude that the glycerol functionality of LGPC is not necessary for the activity, and that the minimum structural features contributing to the activity of these compounds include a medium to long alkyl chain and a zwitterionic group. While these results do not show that a zwitterionic compound containing a short alkyl chain is not very effective as an enhancer of paracellular permeability, a previous report[9] clearly suggests that such is the case.

Effect of DPC on TEER as a Function of Time and Concentration—The influence of DPC on TEER across Caco-2 monolayer was monitored as a function of time at several concentrations (Figure 3). At room temperature, monolayers treated with up to 0.5 mM DPC showed very little change in TEER with only 0−20% drop for the first 30 min. However, when the concentration of DPC was increased to 0.75 mM, a rapid decrease in TEER (within 3 min) was observed. The drop in TEER as a function of concentration exhibited a sharp inflection between 0.5 mM and 0.75 mM of DPC. Interestingly, the drop in TEER as a function of concentration is more gradual and uniform at 37 °C (data not shown) than at room temperature. To determine whether the effect of DPC on Caco-2 cell monolayers is reversible, apical HBSS containing DPC was replaced with fresh cell culture medium after 30 min of treatment. In all cases, TEER returned to the control value upon removal of DPC when measured after 10 h. This indicates that DPC treatment does not lead to the disruption of the monolayer or cell lysis (see cytotoxicity of tight junction modulators).

The effect of DPC on TEER was much more pronounced when it was applied to the apical side than to the basolateral side of Caco-2 cell monolayers (Figure 3 insert). Furthermore, the decrease in TEER achieved after baso-

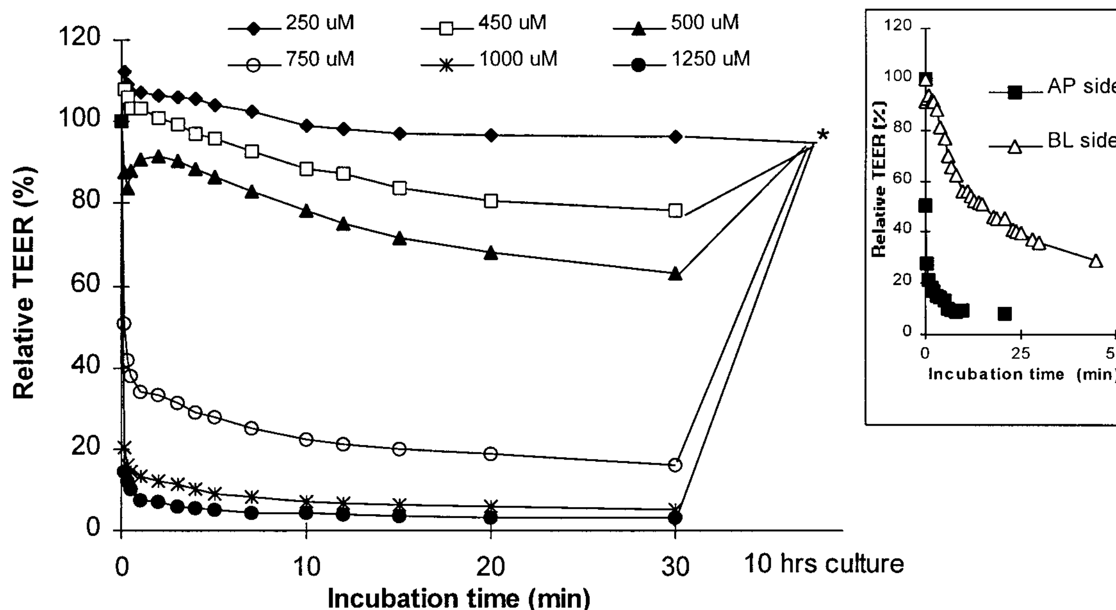

Figure 3—Effect of DPC on TEER (ambient temperature) across Caco-2 cell monolayers as a function of time and DPC concentration. In all cases TEER values returned to the control value when measured 10 h after removal of DPC (after 30 min treatment). The insert displays the effect of 1.0 mM DPC on TEER when it was applied to AP or BL side of the cell monolayers.

lateral application of DPC was much more gradual than that achieved after apical application; in fact, the magnitude of maximum decrease in TEER after basolateral application of DPC never approached that seen with the apical application. We have confirmed that the delayed and decreased effect of DPC when applied on the basolateral side is not due to the polycarbonate membrane, as it does not constitute a significant barrier to the transport of DPC (data not shown). The asymmetry in the effect on TEER suggests that the effect of DPC on TEER or mannitol permeability is not due to its permeation into the cells, followed by an intracellular change. Rather, the effect appears to be due to different interactions with the tight junction components depending on whether DPC is on the apical or the basolateral side. It is also possible that the observed difference in the effect on the decrease in TEER is due to interactions of DPC with cell membrane on the apical versus the basolateral side that lead to different effects on the structure and function of the tight junctions.

Effect of DPC on the Transport of Paracellular Markers—To determine if DPC-induced drop in TEER is accompanied by an increase in the tight junction permeability, we measured transport rates of a few paracellular markers across the Caco-2 cell monolayers. The enhancing effect of DPC pretreatment of the cell monolayers on the transport of hydrophilic paracellular markers was concentration-dependent (Figure 4). At low concentration of DPC (less than 0.5 mM), the permeability of all paracellular molecules was the same as that of the control. In general, DPC-induced decrease in TEER correlated with an increase in transport of the paracellular markers. At 0.75 mM concentration (a nontoxic concentration, see cytotoxicity of tight junction modulators), DPC pretreatment increased the apparent permeability constant (Papp) across Caco-2 cells for mannitol by 12-fold, for LY by 16-fold, and for PEG-900 by 2.2-fold. It caused no significant enhancement in the Papp value for PEG-3500, a large MW paracellular marker. Furthermore, DPC did not affect the transport rate of testosterone, a transcellularly transported marker, indicating that cell membranes were not compromised or disrupted by DPC at 0.75 mM concentration.

The enhancement in permeability of paracellular molecules is treatment-dependent. When DPC was coadministered with the paracellular markers after the initial pretreatment by DPC for 20 min (0.75 mM), further enhancement in the transport rates was observed for these compounds. Especially the transport of PEG-3500, a larger molecular weight marker, could be effectively enhanced with pre- plus cotreatment with DPC (data not shown).

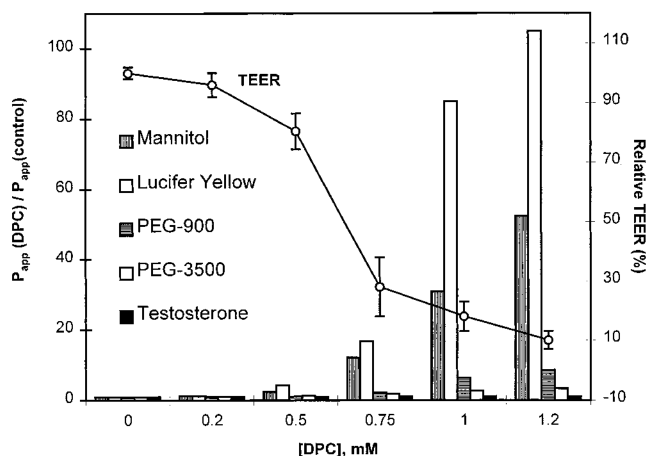

Figure 4—Enhancement of transport of paracellular markers by DPC after 20 min pretreatment. Testosterone was included as control (marker for transcellular transport) to ensure that the cell membrane was not disrupted and that the integrity of the cell monolayer was not compromised. ○: relative TEER (%).

Comparison of Potency of the Tight Junction Modulators—The absorption enhancers are used at different concentrations and in different model systems by different groups. Because of this, it is impossible to establish the concentration at which a given enhancer is effective or to determine (from the literature) relative potencies of different enhancers. Hence, we define the term EC_{50}, the concentration at which an enhancer decreases the TEER of the cell monolayer to 50% of the initial (control) value, and propose that the enhancers should be tested at several concentrations in order to define their effective concentration(s) and their relative potencies. This will allow a more meaningful translation of the results from different groups and comparison of relative potencies of different enhancers. While TEER at a given value may not

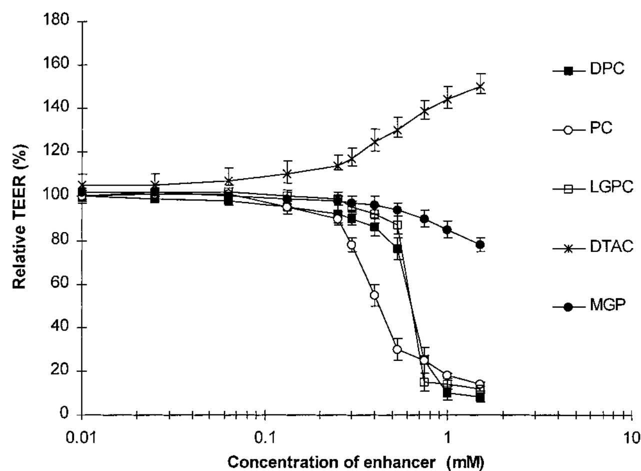

Figure 5—Relationship between concentration of the transport modulators and TEER. The cell monolayers were treated with the compounds for 20 min at ambient temperature. The measurements were made in triplicate and expressed as mean ± SD.

be indicative of the paracellular permeability of compounds, the changes in TEER over a range of the enhancer concentrations correspond well with the changes in paracellular permeability.[28] Therefore, the EC_{50} values provide a good indicator of the potency of a given compound in causing an increase in paracellular permeability. Such an approach has been previously used by Anderberg et al.[29] to compare the efficacy of surfactant excipients. The effect of five agents on TEER is compared as a function of concentration in Figure 5. The EC_{50} values for PC (0.42 ± 0.06 mM), LGPC (0.65 ± 0.04 mM), and DPC (0.65 ± 0.05 mM) indicate that DPC and LGPC are equipotent, with PC showing a somewhat higher potency than the other two compounds. Interestingly, the EC_{50} value for DPC increased from 0.65 mM to 0.74 mM when the temperature increased from 20 °C to 37 °C (data not shown). The anionic MGP was significantly less effective as it caused less than 20% drop in TEER at concentration as high as 1.5 mM. The cationic compound DTAC actually caused a concentration-dependent increase in TEER at concentrations above 0.2 mM. Similar increase in TEER by cationic compounds has been observed previously.[30,31]

Cytotoxicity of the Tight Junction Modulators—The reversibility of the effect of DPC on TEER across Caco-2 cell monolayers (Figure 3) suggests that its effect is not secondary to cytotoxicity resulting in disruption of the cell monolayer. To further evaluate the cytotoxicity of these enhancer-related agents toward Caco-2 cells, the viability of the cells was directly measured using the MTT test.[25,26,29] MTT is a tetrazolium salt that is oxidized by mitochondrial dehydrogenases in living cells to give a dark blue formazan product. Damaged or dead cells show reduced or no dehydrogenase activity. Reports of this and other related tests to assess the cytotoxicity of absorption enhancers toward Caco-2 cells have appeared recently.[20−24,26,29] The results depicted in Figure 6 indicate that at 0.1 mM concentration, none of the agents were cytotoxic. However, at higher concentrations (more than 0.2 mM), the toxicity caused by the agents showed significant differences. To quantitatively compare the cytotoxicity of the enhancer-related agents, the IC_{50} values (concentration at which the enhancer produces 50% inhibition of the mitochondrial dehydrogenase activity measured by the MTT method) were determined. DPC (IC_{50} = 0.92 ± 0.03 mM) and LGPC (IC_{50} = 0.84 ± 0.02 mM) were approximately 2-fold less cytotoxic than PC (IC_{50} = 0.45 ± 0.05 mM) toward Caco-2 cells. SDS, used as a positive control, had an IC_{50} value of ~0.2 mM (data not shown). It

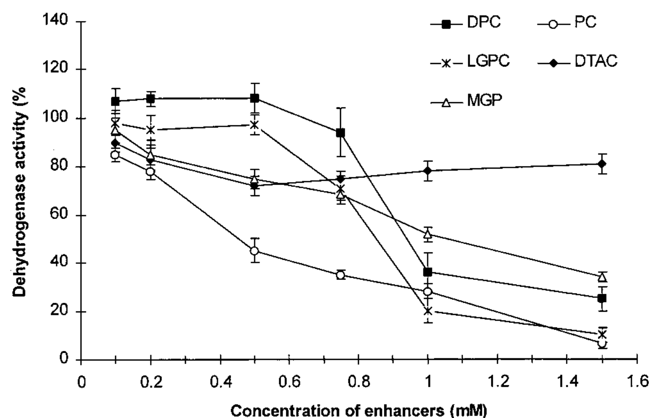

Figure 6—Mitochondrial dehydrogenase activity (MTT) in Caco-2 cells after treatment with transport modulators at various concentrations. All measurements were made in triplicate and expressed as mean ± SD.

is interesting to note that for the extensively used absorption enhancer PC there is no separation between its EC_{50} and its IC_{50} value. Both DPC and LGPC are likely to be somewhat better as absorption enhancers than PC, as their IC_{50} values are ~50% greater than their EC_{50} values. It is important to note that the "cytotoxic" effect of DPC (and other agents) reported here may actually be "cytostatic" effect as evidenced by the fact that removal of DPC from the solutions bathing the cell monolayers results in restoration of TEER over several hours.

Effect of DPC on the Apical Cell Membrane Integrity—To ensure that the effect of DPC and related enhancers was predominantly due to modulation of the tight junctions and not due to compromised apical membrane, we have evaluated DPC-induced DNA staining by propidium iodide. This probe fluoresces when it interacts with DNA, but can access DNA only if the cell membrane and the nuclear membrane are compromised, as it does not permeate intact membranes.[32] When Caco-2 cells were treated with different concentrations of DPC prior to propidium iodide staining, fluorescence microscopy showed that the permeability of the cell membrane remained low after 20 min of exposure to 0.75 mM DPC, but increased at 1.0 mM and 1.25 mM DPC exposures (Figure 7). After removal of DPC and incubation in cell culture medium for 8 h, the membrane integrity was restored for 0.75 and 1.0 mM DPC treatment, but not after treatment with 1.25 mM DPC. These results suggest that above 1 mM concentration, DPC causes irreversible damage to the cell membrane and/or the nuclear membrane.

Effect of DPC on ZO-1 Redistribution—ZO-1 is a tight junction-associated protein,[33,34] which is localized on the cytoplasmic surface just beneath the membrane. When Caco-2 cells are treated with a rabbit antibody to ZO-1 (and visualized by treatment with FTIC-labeled goat anti-rabbit IgG antibody), the fluorescence image obtained with a confocal microscope clearly indicates a uniform distribution of ZO-1 at intercellular junctions (Figure 8A). Interestingly, treatment of these cells with DPC (0.75 mM) appears to cause a redistribution of ZO-1 as indicated by discontinuous ZO-1 labeling (Figure 8B). Thus, DPC appears to affect the spatial distribution of ZO-1 in Caco-2 cells by inducing its dissociation from the plasma membrane.

Discussion

To date, numerous compounds have been evaluated and used as absorption enhancers for orally administered drugs.[3−20] These enhancers fall in one of two categories: Ca^{2+} chelators that act by reducing the extracellular Ca^{2+}

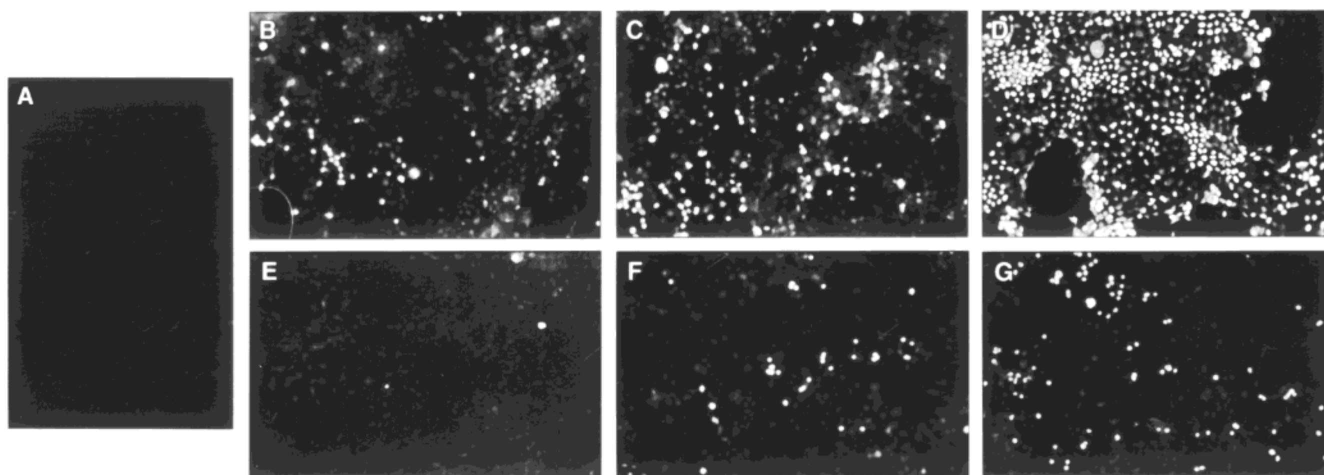

Figure 7—Effect of DPC treatment on the permeability of Caco-2 cells to propidium iodide. Caco-2 cells were treated with propidium iodide; control cells exclude propidium iodide and show no nuclear fluorescence (A). Cells were exposed for 20 min to 0.75 mM (B, E), 1.0 mM (C, F), and 1.5 mM (D, G) DPC. Propidium iodide was applied immediately after DPC treatment (B, C, D) or after DPC was removed and the cells were allowed to recover for 8 h (E, F, G). The frames are photographs of the images obtained by fluorescence microscope using 10x objective.

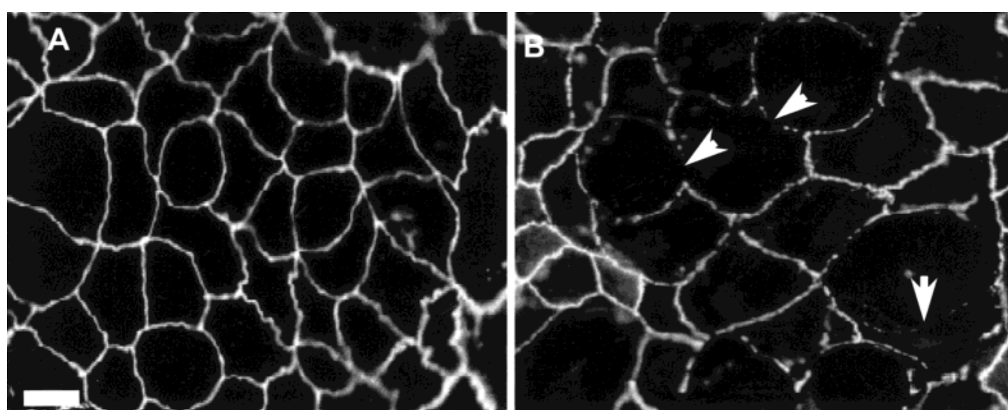

Figure 8—Effect of DPC treatment on the distribution of the tight junction-associated protein ZO-1 in Caco-2 cells. Untreated cells (A) or those treated with 0.75 mM DPC (B) were allowed to react sequentially with ZO-1 antibody and FITC-labeled anti-rabbit IgG as described in the Methods Section, and examined with a fluorescence confocal microscope. Bar = 50 μm.

concentration and thus compromising the integrity of the tight junctions, and detergents/surfactants that may act by causing a perturbation in the lipid bilayer of cell membrane as well as by a variety of intracellular mechanisms.[15−18,22,23] Among the latter class of compounds, PC has been one of the most extensively studied and used absorption enhancers. It appears to function by increasing the intracellular concentration of Ca^{2+} ions; however, the exact mechanism of its action is yet to be understood.[15,17] Also included in the latter class of compounds are phospholipid derivatives such as lysophosphatidylcholines[9,35,36] that appear to increase the permeability of peptides such as human growth hormone and a vassopressin derivative by unknown mechanism(s). These compounds have several features that make them particularly attractive as absorption enhancers. They occur in biological membranes as products of phospholipid metabolism[8,9] and are further metabolized to relatively nontoxic endogenous products. Furthermore, their absorption-promoting effects are spontaneously reversible upon their removal.

In the present study we have demonstrated that DPC, a zwitterionic amphiphile that lacks the glycerol moiety of lysophosphatidylcholine, is equally effective as the lysophosphatidylcholine LGPC in improving the permeability of hydrophilic compounds across Caco-2 cell monolayers. We have further showed that the presence of an (medium to long) alkyl chain and a zwitterionic functionality are essential for its activity. Thus, our studies have defined the minimum structural requirements of the phospholipid derivatives for their activity as absorption enhancers. It is interesting to note that while PC is a member of a different chemical class than phospholipids, its structural features essential for absorption-enhancing activity are quite similar to those for DPC; i.e., presence of an alkyl chain and a zwitterionic functionality (see Figures 1, 2). This is an important finding as the structures of DPC and PC can form the basis of a systematic structure−activity relationship that could lead to a more definitive information regarding the biochemical mechanism underlying their activity as absorption enhancers.

Our studies have shown that DPC, like PC, increases the permeability of the Caco-2 cell monolayers toward hydrophilic compounds predominantly by modulation of the tight junctions and not by disruption of the cell membrane. This was evidenced by a decrease in TEER and an increase in the permeability of paracellular markers (e.g., mannitol, LY, etc.) with no change in the permeability of the transcellular transport marker testosterone. While the cell membrane was rendered somewhat permeable to propidium iodide at high concentration of DPC, it was clear that DPC did not cause extensive damage to the cell membrane at the concentrations that caused significant decease in TEER and an increase in mannitol permeability. Furthermore, our evidence that DPC caused significant redistribution of the tight junction-associated protein ZO-1 (Figure 8) provided confirmation that this compound

increases the permeability of Caco-2 cell monolayers by modulation of the tight junctions.

Our results show that DPC causes a rapid decrease in TEER as has been observed for other agents that act via modulation of tight junctions, such as PC[15] and sodium caprate.[16] The decrease in TEER is more gradual and less extensive when DPC is applied from the basolateral side, suggesting that the effect on the apical side is due to modulation of the tight junctions directly or through perturbation in the apical membrane that is not as accessible from the basolateral side due to the presence of the tight junctions. The decrease in TEER and increase in the permeability of the paracellular markers caused by DPC was concentration dependent, with a sharp inflection occurring (for drop in TEER) between 0.5 and 0.75 mM concentration (Figure 3). The inflection did not correspond to a change in solution properties of DPC; for example, the critical micelle concentration (CMC) for DPC was found to be 0.96 mM. In fact, our results suggested that the effect on TEER was not related to the CMC of these agents, as CMC for PC was more than 1 order of magnitude lower (0.015 mM) than that for DPC despite their somewhat similar potency in causing a decrease in TEER. When the Caco-2 cell monolayers were exposed to DPC, LGPC, and PC at concentrations above CMC for 20 min and then washed, the TEER values rapidly decreased and the transport rates of mannitol were also significantly enhanced compared to the control (data not shown). These results suggest that the monomer and the micelle, at least for these agents, are both active as modulators of the tight junctions.

It has been shown previously[14] that treatment with paracellular enhancers such as EGTA or PC causes a greater enhancement in the permeability of anionic or cationic compounds than of the neutral molecules. Our results confirm this finding in that the permeability of anionic LY was enhanced to a greater extent than that of neutral mannitol by pretreatment of Caco-2 cell monolayers with DPC (Figure 4). As expected, the enhancement in the permeability of the larger neutral molecules (PEG-900 and PEG-3500) is much smaller than that achieved with a smaller neutral molecule like mannitol (Figure 4).

It is important that we are able to compare different absorption enhancers not only in terms of their potency in causing an increased transport of drug molecules, but also in terms of the separation between their potency as enhancers and their potency in causing cellular toxicity. Hence, we propose the use of the parameter PI (potency index) that is a ratio of IC_{50} and EC_{50}, where IC_{50} refers to the concentration that causes a 50% decrease in mitochondrial dehydrogenase activity (an indicator of cytotoxicity in MTT test) and EC_{50} refers to the concentration that causes 50% decrease in TEER with respect to untreated control. We have used TEER as a surrogate for permeability of a paracellular marker because it is an easy measurement and EC_{50} values for TEER relate well to the similar index for permeability of a paracellular marker.[28] While it has been reported frequently that a change in TEER does not parallel a change in the permeability of paracellular markers, when these effects are measured over a concentration range and expressed as relative change they do indeed relate well with each other as has been shown.[28] The potency index for DPC is 1.42; for LGPC, 1.29; for PC, 1.07; for SDS, 1.11; and for MGP, less than 0.22. The larger potency index would mean a better separation between the activity and cytotoxicity of an enhancer, and thus a greater potential for an agent to be useful as an absorption enhancer. DPC possesses better separation between activity (tight junction modulation) and cytotoxicity than all other absorption enhancers evaluated, although less than 2-fold separation is not adequate for safe use of such an agent as an absorption enhancer. Interestingly, for one of the more extensively used agent PC, the potency index is approximately 1, indicative of no separation between toxic and effective concentrations. Clearly, the relationship between such parameters obtained in vitro to the activity and toxicity of such agents in vivo needs be evaluated extensively before the in vitro parameters can be used to compare different enhancers. However, use of such parameters should make the comparison of enhancers more meaningful when they have been evaluated in different groups and in different model systems rather than reporting the activity at one or two arbitrary concentrations. The comparisons of the efficacy of absorption enhancers evaluated by different groups can be made even more meaningful if one of the enhancers is used as a reference compound so that the potencies of the other enhancers can be expressed in relation to the EC_{50} of the reference compound.

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