Structure−Activity Relationship for Enhancement of Paracellular Permeability across Caco-2 Cell Monolayers by 3-Alkylamido-2-alkoxypropylphosphocholines

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Paracellular permeability enhancers have been used to improve the oral bioavailability of hydrophilic drugs; however, the mechanism of action of many enhancers is poorly understood. In this study, highly potent enhancers of paracellular permeability were identified in the 3-alkylamido-2-alkoxypropylphosphocholine series, and a structure−activity relationship was developed for enhancement of paracellular permeability across Caco-2 cell monolayers. Compounds with short (<5 carbons) hydrocarbon chains at both C-2 and C-3 were generally inactive. The potency exhibited a parabolic relationship with respect to the chain length at either C-2 or C-3. Linear molecules (i.e., compounds with a short hydrocarbon chain at C-2 or C-3 and a long hydrocarbon chain on C-3 or C-2, respectively) were more potent than the corresponding branched molecules with the same carbon load. The efficacy of 3-alkylamido-2-alkoxypropylphosphocholines as enhancers of paracellular permeability was not dependent on their existence in micellar form or their ability to alter the fluidity of cell membrane. Previously, a correlation between the potency of alkylphosphocholines as enhancers of paracellular permeability and the inhibitors of phospholipase C (PLC) was established in Madine Darby canine kidney (MDCK) cell monolayers. The potencies of selected 3-alkylamido-2-alkoxypropylphosphocholines as inhibitors of PLC and enhancers of paracellular permeability fit well into this correlation. Therefore, phosphocholines are likely to increase paracellular permeability by modulating the signal transduction pathway initiated by a PLC-catalyzed reaction rather than by physically altering the cell membrane.

Introduction

The intestinal epithelium constitutes a major barrier to oral drug delivery.[1] Molecules cross the intestinal epithelium into the systemic circulation primarily by three pathways: passive diffusion across the cell membranes (transcellular pathway), passive diffusion between adjacent cells (paracellular pathway), or carrier-mediated transport (carrier-mediated transcellular pathway).[2] Lipophilic molecules easily cross the cell membrane via transcellular diffusion. On the other hand, hydrophilic molecules, if not recognized by a carrier, cannot partition into the hydrophobic membrane, and thus traverse the epithelial barrier via the paracellular pathway. The transport of hydrophilic molecules via the paracellular pathway, however, is severely restricted by the presence of tight junctions.[3]

In recent years, the morphology and regulation of the tight junction has been studied in great detail. The tight junction allows passage of small hydrophilic compounds—ions, nutrients, and drugs—but acts as a barrier to larger hydrophilic ones.[4,5] It also acts as an intramembrane diffusion barrier that restricts the intermixing of apical and basolateral membrane components,[6,7] and thus maintains polarity of enterocytes.[8] The restricted movement of ions across the tight junctions gives rise to transepithelial electrical resistance (TEER), which is often used as an index of the integrity of the tight junctions in an epithelial or endothelial tissue. The function of the tight junction is complex; this complexity is also reflected in its multiprotein architecture that spans extracellular, transmembrane, and intracellular domains of the cells. The tight junction is composed of a group of both transmembrane and cytosolic proteins that interact not only with each other but also with the membrane and cytoskeleton.[9]

Many useful therapeutic agents (e.g., peptides, certain cephalosporin antibiotics, etc.) are hydrophilic[10−14] and thus cannot be delivered via the oral route, which is the most favored route of drug delivery. One way to increase the absorption of hydrophilic drugs across the intestinal epithelium is to make them more lipophilic by designing a prodrug, thus increasing transcellular flux of the drug.[11,13] Another approach involves redesigning the drug so that it is a substrate for a carrier (or linked to a substrate for a carrier).[10,14] The controlled and reversible opening of the tight junction represents an alternative approach to increase the absorption of hydrophilic drugs across the intestinal epithelium.[12,15,16] This approach is attractive because it could be applied to many

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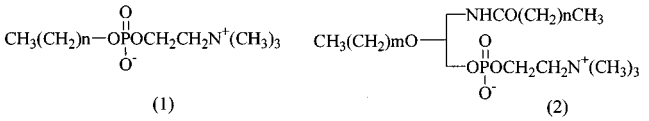

Figure 1. Structures of (**1**) alkylphosphocholines and (**2**) 3-alkylamido-2-alkoxypropylphosphocholines.

different hydrophilic drugs. In addition, this approach would avoid degradation of the active agent by intracellular enzymes, another barrier for absorption of drugs. In recent years, identification of compounds that selectively open the tight junction has been actively pursued.[4] Coadministration of these compounds with hydrophilic drugs improves the oral bioavailability of these drugs in vivo. For example, palmitoyl carnitine increased the oral bioavailability of cefoxitin from less than 5% to as much as 70% in rats.[17] Similarly, a medium chain glyceride increased the absorption of cefmetazole sodium from 5.6 to 64.8% across dog duodenum.[18]

Several phospholipids have been used to increase paracellular permeability. Lysolethicin, a lumenal digestive product of dietary phospholipids and a common component of intestinal lumen, has been explored for use in enhancing oral drug delivery. Talbot et al.[19] reported that relatively low concentration of lysolethicin (0.05−0.20%) increased horseradish peroxidase penetration in guinea pig proximal jejunum without morphological damage. Similarly, alkylphosphocholines (Figure 1) have been found to effectively increase paracellular permeability of hydrophilic compounds across Caco-2 cell monolayers.[20] Furthermore, a limited structure−activity relationship (SAR) study with a series of 3-alkylamido-2-alkoxypropylphosphocholines (Figure 1) indicated that it was possible to vary potency of these compounds as paracellular permeability enhancers by varying the alkyl chain length at the C-2 (alkoxy) or C-3 (alkylamido) positions.[20] In the present study, we report a comprehensive SAR for the 3-alkylamido-2-alkoxyphosphocholines as enhancers of paracellular permeability across Caco-2 cell monolayers, identify potent permeability enhancers, and establish that phosphocholines containing linear alkyl chains are much more potent than the corresponding derivatives with branched chains. In addition, this study also provides evidence that these compounds increase paracellular permeability via specific modulation of the tight junctions, and not by nonspecific perturbation of the cell membrane.

Chemistry

3-Alkylamido-2-alkoxypropylphosphocholines were synthesized in six steps as shown in Figure 2[21] with necessary modifications as noted below. 3-Alkylamido-1,2-propanediols (**4**) were synthesized by reacting commercially available 3-amino-1,2-propanediol (**3**) with an appropriate acid chloride or anhydride. For acid chlorides with more than eight carbons, pyridine/dimethylformamide (DMF) at room temperature gave acceptable yields (method A, 47−70%). With acid chlorides containing fewer than eight carbons, these conditions failed to produce the desired product; however, sodium carbonate in water/chloroform (method B) gave compounds in good yields.[22,23] The water soluble 3-ethanamido-1,2-propanediol was synthesized by using acetic anhydride and sodium carbonate in methanol (method C).[24,25]

The primary hydroxyl group of **4** was then protected as the trityl ether (**5**).[26] Any reaction at the secondary hydroxyl group was minimized by avoiding long reaction times (>10 h) and high temperatures (>50 °C). Next, the secondary hydroxyl group was alkylated with an alkyl bromide and NaH in tetrahydrofuran (THF). The resulting compounds (**6**) were detritylated with *p*-toluenesulfonic acid in CH_2Cl_2/methanol to give the 1-alkylamido-2-alkoxypropanols (**7**).

The final phosphocholines (**2**) were prepared by two methods. Method D was a one pot synthesis of Jia et al.[27] using 2-chloro-1,3,2-dioxaphospholan-2-one and trimethylamine gas. However, in our hands, acceptable and more dependable yields were obtained with method E, a two step approach with 2-bromoethyl dichlorophosphate (**9**) and aqueous trimethylamine.[28] All final products were characterized by nuclear magnetic resonance (NMR) and high-resolution mass spectroscopy (HRMS).

Biology

Effect of 3-Alkylamido-2-alkoxypropylphosphocholines on Paracellular Permeability across Caco-2 Cell Monolayers. In this study, the target compounds have been evaluated as enhancers of paracellular permeability across intestinal epithelium. Caco-2 cell monolayers grown in a transwell, a well-accepted model for intestinal epithelium, were used to evaluate the potency and selectivity of the test compounds as paracellular permeability enhancers. This model was previously used to assess the potency and mechanism of paracellular permeability enhancers of similar compounds.[20,29] In addition, the effect of these compounds on paracellular permeability was also assessed in Madine Darby canine kidney (MDCK) cell monolayers, particularly in experiments relating the enhancer activity to inhibition of phospholipase C (PLC) (cf. section on Determination of Adenosine 3′,5′-Triphosphate (ATP)-Stimulated PLC Activity in MDCK Cells). MDCK cell monolayers, like Caco-2 cell monolayers, have also been used as an in vitro model for assessment of transport rates and transport mechanisms of compounds across epithelial tissues, including intestinal epithelium.[30,31] The effect of target compounds on paracellular permeability was determined by measurement of the decrease in TEER and/or the increase in mannitol (a paracellular marker that traverses the epithelial tissue predominantly via the paracellular route) flux from apical to basolateral (absorptive direction) across Caco-2 cell monolayers.

Effect on TEER. The effect of 3-alkylamido-2-alkoxypropylphosphocholines on TEER was evaluated by a 20 min treatment of the Caco-2 cell monolayers on the apical side with the compounds.[20] The treatment time was determined based on our previous studies[20,32] with structurally similar compounds, which showed that the drop in TEER was maximal after a 20 min treatment. These studies have also shown that the effect of these compounds is reversed over several hours in a static cell culture system. The active compounds decreased TEER in a concentration-dependent manner from a control

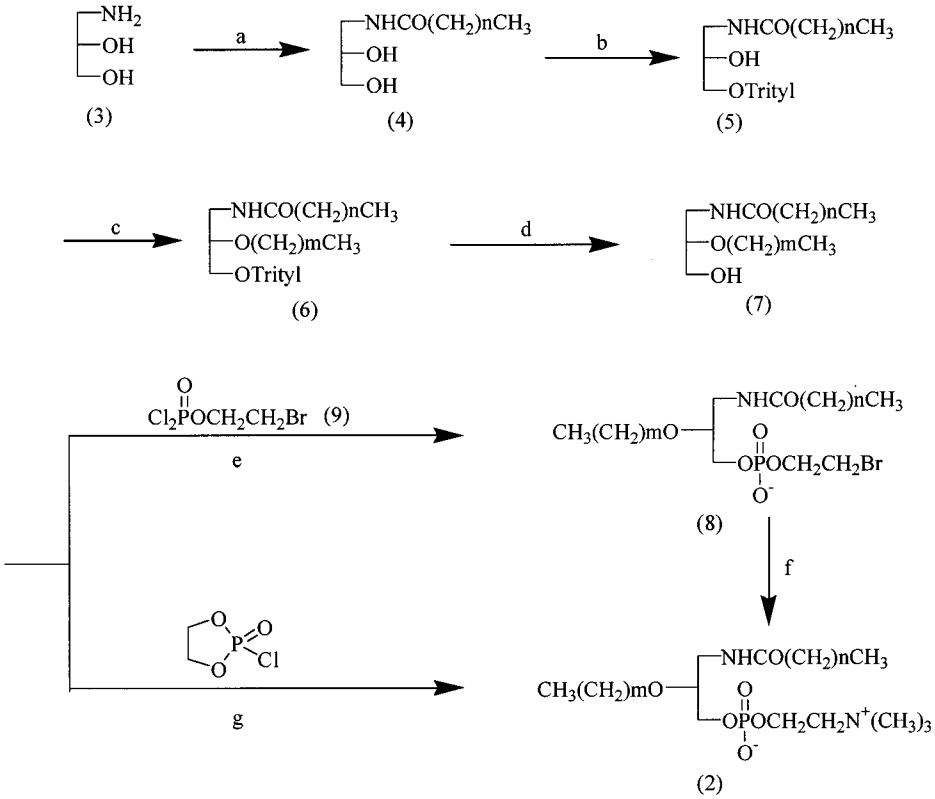

Figure 2. Synthesis of 3-alkylamido-2-alkoxypropylphosphocholines. Reagents: (a) $CH_3(CH_2)nCOCl$/pyridine/DMF; (b) Ph_3CCl/pyridine; (c) $CH_3(CH_2)mBr$/NaH/THF; (d) *p*-toluenesulfonic acid/CH_3OH/CH_2Cl_2; (e) ethyl ether/THF/pyridine; (f) triethylamine (40% aqueous solution)/DMF/$CHCl_3$/isopropanol; (g) triethylamine/acetonitrile.

value of 600−800 Ω·cm^2 down to 20−100 Ω·cm^2. The drop in TEER occurred over a 3−4-fold concentration range for the most potent compounds and over a broader concentration range for less potent compounds. The potency of 3-alkylamido-2-alkoxypropylphosphocholines, expressed as the concentration that decreased TEER by 50% of control value (EC_{50}), varied markedly (Table 1). Compounds with a short alkyl ether chain at C-2 (4−6 carbons) and a short alkylamido chain at C-3 (4−8 carbons) were inactive. Similarly, compounds with moderate to long hydrocarbon chains on both C-2 and C-3, such that the total carbons exceed 22, were also inactive. In the 2-ethoxy series (compounds **10**−**15**), the compound with the C-3 alkylamido group containing 10 carbons was practically inactive with the next higher homologue showing modest activity. However, the potency jumped dramatically when the C-3 alkylamido chain length increased from 12 to 14 carbons; no further increase in potency was observed as the length of the alkylamido chain increased from 14 to 18 carbons, and the potency dropped when the alkylamido chain length increased further to 20 carbons. A similar trend was observed when the 3-alkylamido functionality was kept short (two carbons) and the length of the 2-alkoxy functionality was varied (compounds **32**, **37**, and **41**). For a series in which the alkylamido functionality was fixed at 10 carbons with increasing number of carbons in the 2-alkoxy functionality (carbons 2−16, compounds **10**, **18**, **22**, **25**, **29**, **33**, **36**, and **39**), the potency exhibited a bell-shaped relationship with the maximum potency observed for compounds with 6−8 carbons in the 2-alkoxy functionality. It appears that compounds with a total of 16−20 carbons in the C-2 and C-3 substituents show maximum potency. However, in this subset, the compounds that are branched (i.e., approximately equal numbers of carbons in C-2 and C-3 substituents) have a much lower potency than those that are more linear (i.e., either C-2 or C-3 is very short, i.e., a two carbon chain length).

Effect on Mannitol Flux. As expected, 3-alkylamido-2-alkoxypropylphosphocholines increased mannitol flux in a concentration-dependent manner. The potency of these compounds to increase tight junction permeability was determined by the concentration that increased the mannitol flux by 10-fold (EC_{10x}) of the control value.[20,32] The potency for causing the increased mannitol flux varied with changes in the length of the alkoxy and alkylamido substituents in a manner similar to that observed when TEER was used as an indicator of tight junction permeability, except that generally greater concentrations were required to cause a 10-fold increase in mannitol flux than those required to cause 50% decrease in TEER (Table 1).

Relationship between Critical Micelle Concentration (CMC) and Enhancement of Paracellular Permeability by 3-Alkylamido-2-alkoxypropylphosphocholines. Because of the amphiphilic nature of 3-alkylamido-2-alkoxypropylphosphocholines, they could interact with the cell membrane; thus, it is conceivable that the enhancement of paracellular permeability by this class of compounds is due to compromised integrity of the tight junctions caused by perturbation of the cell membrane. If this were the case, it would be reasonable to speculate that efficacy of these compounds as paracellular permeability enhancers may be related to their ability to form micelles. Hence, the CMC of a selected

Table 1. EC_{50}, EC_{10x}, and Potency Index of 3-Alkylamido-2-alkoxypropylphosphocholines in Caco-2 Cell Monolayers[a]

compd	chain length at C2, C3[b]	EC_{50} mM	EC_{10x} mM	potency index IC_{50}/EC_{50}
10	02, 10	>5.00	>5.00	NA[c]
11	02, 12	1.79 (0.45)	>5.00	1.22
12	02, 14	0.08 (0.00)	0.89 (0.27)	2.00
13	02, 16	0.05 (0.03)	0.30 (0.12)	2.20
14	02, 18	0.04 (0.04)	0.26 (0.03)	4.00
15	02, 20	0.28 (0.01)	0.42 (0.01)	0.89
16	04, 04	>5.00	>5.00	NA[c]
17	04, 08	>5.00	>5.00	NA[c]
18	04, 10	3.19 (0.04)	>5.00	1.24
19	04, 12	0.31 (0.06)	1.87 (0.50)	1.55
20	04, 16	0.09 (0.02)	0.48 (0.08)	1.11
21	06, 08	>5.00	>5.00	NA[c]
22	06, 10	0.42 (0.03)	0.73 (0.05)	2.38
23	06, 12	0.19 (0.11)	1.01 (0.24)	0.84
24	06, 16	0.52 (0.40)	1.61 (0.68)	0.60
25	08, 10	0.30 (0.08)	1.21 (0.20)	1.27
26	08, 12	0.55 (0.40)	1.19 (0.30)	1.24
27	08, 16	>5.00	>5.00	NA[c]
28	08, 18	>5.00	>5.00	NA[c]
29	10, 10	0.66 (0.14)	1.17 (0.13)	2.12
30	10, 12	1.27 (0.35)	>3.00[d]	1.18
31	10, 16	>2.00[d]	>2.00[d]	NA[c]
32	12, 02	0.78 (0.25)	4.27 (0.67)	1.72
33	12, 10	1.04 (0.11)	>3.00[d]	1.92
34	12, 12	>2.00[d]	>2.00[d]	NA[c]
35	12, 16	>1.00[d]	>1.00[d]	NA[c]
36	14, 10	>2.00[d]	>2.00[d]	NA[c]
37	16, 02	0.07 (0.02)	0.35 (0.23)	1.43
38	16, 06	0.30 (0.21)	1.35 (0.04)	0.93
39	16, 10	>2.00[d]	>2.00[d]	NA[c]
40	16, 12	>0.50[d]	>0.50[d]	NA[c]
41	18, 02	0.09 (0.05)	0.34 (0.10)	1.11
42	18, 16	insoluble	insoluble	insoluble

[a] Results are reported as mean ± SD (in parentheses) in triplicate for three separate experiments. [b] See Figure 1, general structure **2**. [c] Not applicable. [d] The compound is insoluble above this concentration.

Table 2. CMC Values of 3-Alkylamido-2-alkoxypropylphosphocholines

compd	chain length at C2, C3	CMC, μM[a,b]
10	02, 10	4700 (110)
18	04, 10	1170 (338)
25	08, 10	55.3 (26.7)
11	02, 12	1030 (222)
12	02, 14	148 (81.5)
13	02, 16	34.7 (2.03)
14	02, 18	10.5 (1.70)

[a] CMC values were determined by fluorescence assay using DPH as a probe. [b] Results are reported as mean ± SD determined in triplicate for three separate experiments.

group of 3-alkylamido-2-alkoxypropylphosphocholines was determined (Table 2) with 1,6-diphenyl-1,3,5-hexatriene (DPH) as a probe,[33] whose fluorescence yield was significantly greater in the lipid environment than in the aqueous environment. The CMC values of 3-alkylamido-2-alkoxypropylphosphocholines decreased 3−5-fold with the addition of every two methylene units on the hydrocarbon chain at either the C-2 or the C-3 position, indicating that the more lipophilic phospholipids form micelles at a lower concentration. These results are consistent with the report by Stafford et al.[34] that the CMC values of 1-acyl-*sn*-glycero-3-phosphocholines decreased 10-fold with the addition of every two methylene units on the acyl chain.

In general, the EC_{50} values varied in the same direction as the CMC values of 3-alkylamido-2-alkoxypropylphosphocholines. However, compounds exhibited activity at concentrations above or below CMC. For example, the EC_{50} values of compounds **14** and **25** were 4- and 6-fold higher than their respective CMC values. On the other hand, the EC_{50} value of compound **12** was approximately half that of its CMC value (Table 2). These results suggested that the efficacy of the phosphocholines as paracellular permeability enhancers is not dependent on their ability to form micelles.

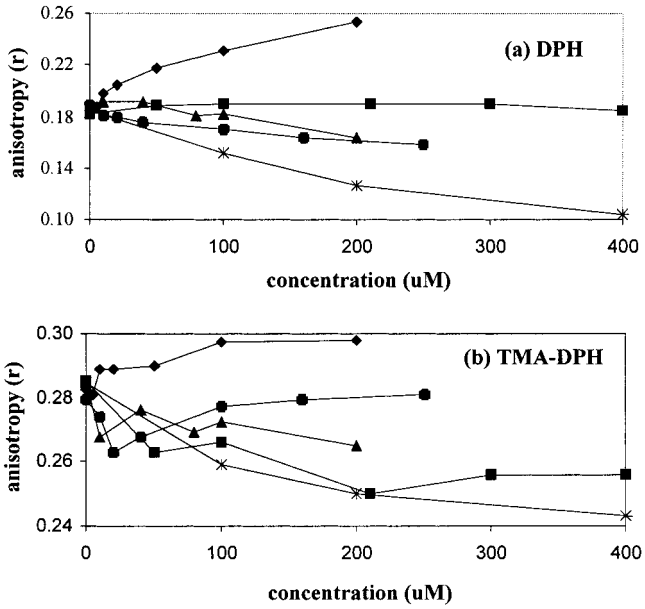

Figure 3. Concentration-dependent effects of selected compounds on the steady state fluorescence anisotropy measured by (a) DPH and (b) TMA-DPH in Caco-2 cells (ambient temperatures). Data represent the means of three measurements with different cell preparations. The standard deviations of data points were 1−4% of means. Symbols that represent the compounds are as follows: ♦, cholesterol; ■, SDS; ▲, HPC; ●, compound **14**; *, compound **25**.

Cytotoxicity of 3-Alkylamido-2-alkoxypropylphosphocholines to Caco-2 Cells. The toxicity of 3-alkylamido-2-alkoxypropylphosphocholines toward Caco-2 cells was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which measures the mitochondrial dehydrogenase activity as a surrogate for cell viability by a colorimetric assay.[45−47] IC_{50} was determined as the concentration that decreases the activity of mitochondrial dehydrogenase by 50% as compared to the control (untreated) value. The potency index (Table 1) was defined as IC_{50}/EC_{50}. For most 3-alkylamido-2-alkoxypropylphosphocholines, the potency index was found to be between 0.5 and 2.0. The only exception was 3-octadecanamido-2-ethoxypropylphosphocholine (compound **14**), with $IC_{50}/EC_{50} = 4.0$ ($IC_{50} = 0.16$ mM, $EC_{50} = 0.04$ mM).

Relationship between the Ability of 3-Alkylamido-2-alkoxypropylphosphocholines to Affect Membrane Fluidity and Cause Paracellular Permeability Enhancement. The effect of 3-alkylamido-2-alkoxypropylphosphocholines on cell membrane fluidity was determined in Caco-2 cells by fluorescence anisotropy (r) measurement with DPH or trimethylammonium-1,6-diphenyl-1,3,5-hexatriene tosylate (TMA-DPH) as probes (Figure 3).[35,36] DPH incorporates into the hydrophobic region of the lipid bilayer; therefore, its fluorescence anisotropy provides a measure of the

average molecular packing order of the hydrophobic regions of the cell membranes.[37] By contrast, TMA-DPH is localized at the lipid−water interface and its fluorescence anisotropy provides a measure of the lipid order at the surface of the cells (i.e., hydrophilic region).[38]

Cholesterol, which is known for increasing the packing order of membranes,[37] significantly decreased the membrane fluidity of both hydrophilic and hydrophobic regions of Caco-2 cell membranes, as indicated by the increased anisotropy observed upon treatment with both DPH and TMA-DPH. In contrast, sodium dodecyl sulfate (SDS), an anionic detergent that increases cellular permeability by damaging cell membranes,[39] significantly decreased the anisotropy measured by TMA-DPH without affecting the fluorescence anisotropy measured by DPH, indicating that SDS selectively increased the fluidity of the hydrophilic region of the cell membrane. These results confirmed that fluorescence anisotropy measured with DPH and TMA-DPH was a good indicator of increased or decreased fluidity of cell membranes with respect to control. One of the potent 3-alkylamido-2-alkoxypropylphosphocholines (compound **14,** linear chain) had little effect on membrane fluidity at its EC_{50} (i.e., 40 μM). In contrast, one of the less potent 3-alkylamido-2-alkoxypropylphosphocholines (compound **25**, branched chain) significantly increased the membrane fluidity in both regions at a concentration near its EC_{50} (i.e., 300 μM). These results suggest that the paracelluar permeability enhancement by 3-alkylamido-2-alkoxypropylphosphocholines is not the direct result of the alteration of the cell membrane fluidity. The results also suggest that the potent 3-alkylamido-2-alkoxypropylphosphocholines may increase paracellular permeability by a mechanism other than grossly altering the structure of cell membranes, while the less potent ones may achieve their effects, at least in part, by interacting with cell membranes. Our results with a related potent enhancer of paracellular permeability, hexadecylphosphocholine (HPC),[40] confirmed this conclusion in that HPC caused only a slight increase in membrane fluidity in the hydrophilic region, and this effect was independent of concentration.

Relationship between Inhibition of ATP-Stimulated PLC and Enhancement of Paracellular Permeability across MDCK Cell Monolayers. We have demonstrated that alkylphosphocholines increase tight junction permeability across MDCK cell monolayers through inhibition of PLC.[40] In addition, PLC has been implicated in the modulation of paracellular permeability by fatty acids and acyl carnitines.[41,42] Alkylphosphocholines are structurally similar to 3-alkylamido-2-alkoxypropylphosphocholines containing a short branch. To determine if this same biochemical mechanism is behind the ability of these compounds to increase tight junction permeability, the effect of two 3-alkylamido-2-alkoxypropylphosphocholines containing a short branch [e.g., 3-tetradecanamido-2-ethoxypropylphosphocholine (compound **12**) and 3-octadecanamido-2-ethoxypropylphosphocholine (compound **14**)] on paracellular permeability and ATP-stimulated PLC activity was determined in MDCK cell monolayers. MDCK cells were used because the relationship between PLC activity and paracellular permeability enhancement has been more extensively investigated in this model than in Caco-2 cells.[40,43,44] On the other hand, both Caco-2 cells and MDCK cells have been used for assessment of transport properties of compounds across epithelial tissue, including intestinal epithelium.[30,31] These compounds, similar to alkylphosphocholines, inhibited PLC and increased paracellular permeability in a concentration-dependent manner. The $IC_{50(PLC)}$, which was defined as the concentration that inhibited ATP-stimulated inositol triphosphate formation by 50%, was 30 ± 17 and 10 ± 4 μM, respectively, for compounds **12** and **14**. The EC_{50} in MDCK cell monolayers for these compounds was 51 ± 9 and 13 ± 4 μM, respectively, and the EC_{10x} was 121 ± 16 and 27 ± 5 μM, respectively. The potencies of these compounds to increase paracellular permeability and inhibit PLC maintained the same rank order, suggesting the existence of an association between inhibition of PLC and increase in tight junction permeability caused by these compounds. In addition, the potencies to cause inhibition of PLC and increase in paracellular permeability of 3-alkylamido-2-alkoxypropylphosphocholines fit very well to the relationship between the potencies of alkylphosphocholines to cause inhibition of PLC and enhancement of paracellular permeability in MDCK cell monolayers (Figure 4), suggesting that inhibition of PLC might be the biochemical mechanism underlying the ability of 3-alkylamido-2-alkoxypropylphosphocholines to increase paracellular permeability.

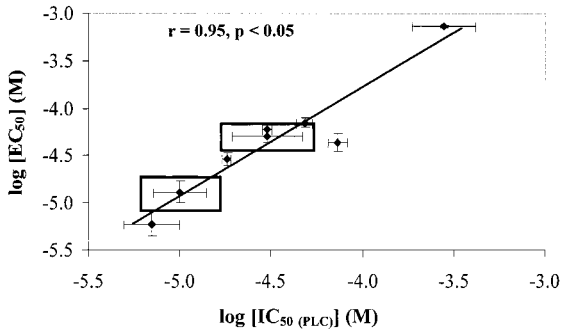

Figure 4. Correlation between the $IC_{50(PLC)}$ and the EC_{50} values for U73122 (a potent PLC inhibitor), five alkylphosphocholines (decylphosphocholine, dodecylphoaphocholine, tetradecylphosphocholine, hexadecylphosphocholine, and octadecylphosphocholine), and two 3-alkylamido-2-alkoxypropylphosphocholines (compounds **12** and **14**). The data points for the title compounds are highlighted with rectangles. Data points represent mean ± SD in triplicate from three separate experiments.

Discussion

There are numerous reports indicating that phospholipids and related compounds enhance permeability of tight junctions in epithelial tissue.[4] Hence, many phospholipid derivatives have been investigated as enhancers of oral or nasal delivery of hydrophilic drugs with poor absorption. The choice of these agents as absorption enhancers is frequently empirical, and the mechanism of their action is poorly understood. In a previous paper, we showed that a long alkyl chain (C-12) and a zwitterionic headgroup are sufficient structural features for causing enhancement in tight junction permeability.[20,32] In the accompanying study, we also showed that glycerophospholipid analogues containing a short alkyl ether functionality at C-2 (methoxy or ethoxy) and a

long alkylamido moiety at C-3 (amides of 16−19 carbon acids) were quite potent as enhancers of tight junction permeability across Caco-2 cell monolayers.[20] We further showed that derivatives with longer alkyoxy chain length at C-2 were less potent. The results with a limited series of 3-alkylamido-2-alkoxyphosphocholines suggested that branching at C-2 might be detrimental to the potency of these compounds as enhancers of tight junction permeability. In the present study, the effect of the alkyl chain length at C-2 and C-3 on the potency as enhancers of paracellular permeability is systematically investigated with 32 phosphocholine derivatives. This study represents the first attempt at systematically evaluating the importance of linear vs branched alkyl chains in defining the potency of phosphocholines as enhancers of tight junction permeability. The 3-alkylamido-2-alkoxyphsophocholines were chosen for two reasons: (i) The glycerol skeleton allowed access to synthetic schemes for varying alkyl chain lengths at C-2 and C-3; this enabled us to design compounds with varying extent of branching. (ii) These compounds represent metabolically stable analogues of naturally occurring glycerophospholipids.

The potency of these compounds as enhancers of paracellular permeability varied significantly as the length of the alkyl chains at C-2 and C-3 was varied. With a short alkoxy chain at C-2, the potency exhibited a parabolic relationship with respect to the chain length at C-3. A similar behavior was observed for compounds with a short alkylamido chain at C-3 and varying chain length at C-2. Interestingly, the compounds with a linear configuration (i.e., a short chain at C-2 and a long chain at C-3 or vice versa) were much more potent enhancers of paracellular permeability than those with a branched configuration for the same number of total carbons at the two positions. The potent enhancers identified in this study are at least an order of magnitude more potent than previously known amphiphilic enhancers such as palmitoyl carnitine.[16,17,32]

While EC_{50} and EC_{10x} values of these compounds appear to be related to their CMC values, the compounds are efficacious as enhancers above and below their CMC values, suggesting that the enhancement of paracellular permeability was not simply due to strong interaction of these compounds with the cell membranes in micellar form. Furthermore, the concentrations at which the glycerophospholipid analogues caused changes in the cell membrane fluidity (as evidenced by the changes in fluorescence anisotropy of DPH and DPH-TMA) did not appear to be related to the concentrations at which more potent compounds caused enhanced paracellular permeability. For less potent compounds, changes in membrane fluidity may be contributing to their effect on paracellular permeability. Thus, it is reasonable to conclude that the enhancement of paracellular permeability caused by 3-alkylamido-2-alkoxypropylphsosphocholines is not due to gross perturbation of the cell membrane, but rather, it is due to specific changes in the structure and/or function of the tight junction.

PLC is an important regulatory enzyme that catalyzes hydrolysis of phosphatidylinositol-4,5-bisphosphate into inositol triphosphate and diacylglycerol in response to the stimulation of a variety of receptors, e.g., stimulation of $P2Y_2$ receptors by ATP.[48] The activity of different families of PLC is regulated through different receptor-mediated pathways. For example, the activities of PLC-β and PLC-γ are regulated through G-protein-coupled receptors (e.g., purinergic receptor) and receptor tyrosine kinases (e.g., epidermal growth factor receptor), respectively.[49] The PLC-dependent pathway has been implicated in the assembly of the tight junction,[50−54] particularly during development, although definitive evidence for its role in the function of the mature tight junctions is lacking. HPC, an inhibitor of PLC,[55,56] has structural features (i.e., a long alkyl chain and a zwitterionic functionality) that have been previously identified to cause an increase in tight junction permeability.[20,32] HPC was found to be a potent enhancer of tight junction permeability across MDCK cell monolayers and a potent inhibitor of PLC in these cells. Our recent studies with analogues of HPC, containing alkyl chains of different lengths, established a clear link between inhibitory activity of alkylphosphocholines toward PLC-β and their activity as enhancers of tight junction permeability across MDCK cell monolayers.[40] Additionally, these studies provided evidence that inhibition of PLC-β activity by these compounds increased tight junction permeability via changes in organization of actin filament network. Because the 3-alkylamido-2-alkoxypropylphosphocholines share some of the structural features of alkylphosphocholines, we investigated the possibility that enhancement of tight junction permeability caused by the title compounds is also related to inhibition of PLC-β by these compounds. As shown in Figure 4, not only did these compounds inhibit PLC-β, but the relationship between their potency as PLC-β inhibitors ($IC_{50(PLC)}$) and their potency as enhancers of tight junction permeability (EC_{50}) fit well with the correlation between these two parameters obtained with the alkylphosphocholines. While these results do not prove that the title compounds cause an increase in tight junction permeability via inhibition of PLC-β, they provide a very strong lead for a possible mechanism of action underlying their activity as enhancers of tight junction permeability.

The present study provides some preliminary insights into the structural features of metabolically stable glycerophospholipid analogues that may contribute to the potency of these compounds as enhancers of paracellular permeability. It is encouraging that the enhancement of the paracellular permeability is not due to gross changes in the cell membrane integrity. At least one of the mechanisms by which these compounds may cause enhanced paracellular permeability involves inhibition of the regulatory enzyme PLC. Thus, these compounds will provide leads for identifying mechanism-based approaches to open tight junctions in a controlled and reversible fashion. Such agents are likely to be useful in increasing the oral absorption of hydrophilic drugs, such as peptides, provided that the separation between efficacy and cytotoxicity is significantly improved. The best potency index (IC_{50}/EC_{50}) achieved with this series of compounds was 4, with most of the compounds showing a potency index in the range of 1−2. It would be important to assess the in vivo relevance to this in vitro potency index before significant effort is invested in improving the potency index.

In the intestinal inflammatory diseases such as irritable bowel syndrome, compromised tight junctions are implicated in the etiology of the disease. It is unclear whether the compromised tight junctions are the consequence of the disease or whether they play a role in the progression of the disease. A series of compounds, such as the ones reported in this study, with a well-defined SAR with respect to their ability to cause graded increase in tight junction permeability, could serve as an important mechanistic probe for the construction of experimental models to evaluate the role of the compromised tight junctions in the disease progression.

Experimental Section

General Synthetic Methods. Starting materials were purchased from Aldrich Chemical Co. (Milwaukee, WI) or Fluka Chemical Co. (Milwaukee, WI). Unless otherwise indicated, all of the chemicals were used without further purification. THF was distilled from sodium and benzophenone. Thin-layer chromatography (TLC) was conducted on Aldrich TLC silica gel plates, and fractions were visualized by UV or the spray of either sulfuric acid/methanol (95: 5 v/v) or phosphomolybdate reagent followed by heating. Column chromatography was performed on silica gel 60 (230−400 mesh) and eluted with a mixture of ethyl acetate and hexanes unless otherwise indicated. ^{1}H NMR spectra were recorded on a Varian 300 MHz spectrometer. Chemical shifts were reported in parts per million (δ) relative to tetramethylsilane. Mass spectrum was conducted on a VG Analytical ZAB double-focusing spectrometer. For each typical reaction, only the spectral data of one representative compound are provided (the detailed characterization of the final products is provided in Supporting Information). The identity of the test compounds was established by accurate mass measurement and NMR spectra, and their purity was >98% as evidenced by TLC analysis.

3-Alkylamido-1,2-propanediols (4). Method A. To a magnetically stirred solution of 3-amino-1,2-propanediol (0.32 mol) in 100 mL of pyridine and 350 mL of DMF was added a solution of acid chloride (0.33 mol) in 150 mL of DMF. After 2 h, the total volume of solvent was reduced by three-fourths. The gelatinous mass was filtered, washed with water, and dried in the air. The solid was recrystallized successively from 350 mL of ethanol, 350 mL of 2-propanol, and 100 mL of chloroform to give a white powder.

Method B. A solution of 3-amino-1,2-propanediol (0.17 mol) and K_2CO_3 (0.18 mol) in 120 mL of water was kept on ice-bath. Acid chloride (0.18 mol) in 120 mL of chloroform was added dropwise. After 1 h, the ice-bath was removed and the reaction mixture was kept at 40 °C overnight. The solvent was removed under reduced pressure, and the crude product was filtered to remove the insoluble inorganic salt. The product was obtained as a colorless liquid in quantitative yield.

Method C. A solution of 3-amino-1,2-propanediol (0.10 mol) in acetic anhydride (25 mL) was stirred overnight at room temperature. Acetic acid and acetic anhydride were removed under reduced pressure. Methanol (50 mL) and solid potassium carbonate (10.0 g) were added, and the suspension was stirred for an additional 2 days. The solvent was then removed to give a colorless liquid in quantitative yield. The crude product mixture was used in the next reaction without further purification.

***rac*-3-Hexadecanamido-1,2-propanediol.** Yield, 62%; R_f = 0.60 in chloroform:methanol = 4:1. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.89 (t, 3H, J = 6.8 Hz), 1.26 (broad m, 24H), 1.65 (m, 2H), 2.26 (m, 2H), 3.20−4.20 (m, 5H), 6.12 (m, 1H). MS (FAB): m/z 330.5 (M+1).

3-Alkylamido-1-triphenylmethoxy-2-propanols (5). Trityl chloride (0.11 mol) was added to a stirring solution of 3-alkylamido-1,2-propanediol (0.10 mol) in 250 mL of pyridine. The reaction mixture was kept at 45−50 °C for 10 h. After the pyridine was removed under reduced pressure, the residue was diluted with 100 mL of water and extracted three times with 100 mL of chloroform. The combined extracts were washed with 50 mL each of cold, 5% HCl and saturated NaCl, dried over sodium sulfate, filtered, and evaporated to dryness. The crude residue was recrystallized two times from hexanes giving light yellow products.

***rac*-3-Hexadecanamido-1-triphenylmethoxy-2-propanol.** Yield, 53%; R_f = 0.60 in chloroform. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.89 (t, 3H, J = 6. 9 Hz), 1.27 (m, 24H), 1.57 (m, 2H), 2.09 (m, 2H), 3.10−4.20 (m, 5H), 5.64 (t, 1H, J = 8.1 Hz), 7.20−7.50 (m, 15H). MS (FAB): m/z 572.4 (M+1).

3-Alkylamido-2-alkoxy-1-triphenylmethoxypropanes (6). A solution of 3-alkylamido-1-triphenylmethoxy-2-propanol (0.045 mol) in 100 mL of THF was added to a slurry of 85% NaH (0.05 mol) in 10 mL of THF. After it was stirred for 1 h at room temperature, alkyl bromide (0.05 mol) was added and the reaction mixture was heated to 50 °C for 4 h. An additional 0.01 mol of NaH and 0.02 mol of alkyl bromide was added, and heating was continued overnight. After it was cooled, water was added slowly to decompose any residual NaH. Diethyl ether (100 mL) was added, and the layers were separated. The aqueous layer was re-extracted with ether (2 × 100 mL), and the organic extracts were combined, washed with brine (2 × 100 mL), and dried over sodium sulfate. The crude product was dissolved in hot hexanes, and a small amount of insoluble material was filtered and discarded. The product was purified by silica gel chromatography using hexane:ethyl acetate (9:1 to 4:1).

***rac*-3-Hexadecanamido-2-octadecoxy-1-triphenylmethoxypropane.** Yield, 63%; R_f = 0.30 in hexane:ethyl acetate = 7:1. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.89 (t, 6H, J = 6.5 Hz), 1.27 (broad m, 54H), 1.45−1.70 (m, 4H), 2.09 (t, 2H, J = 7.8 Hz), 3.00−3.70 (m, 7H), 5.77 (t, 1H, J = 5.4 Hz), 7.20−7.50 (m, 15H). MS (FAB): m/z 824.2 (M+1).

3-Alkylamido-2-alkoxy-1-propanols (7). *p*-Toluenesulfonic acid (0.08 mmol) was added to a solution of 3-alkylamido-2-alkoxy-1-triphenylmethoxypropane (0.32 mmol) in methylene chloride (10 mL) and methanol (0.5 mL). The solution was stirred for 8 h at room temperature. Aqueous solution of saturated sodium bicarbonate (2 mL) was added and stirred for 0.5 h. The layers were separated, and the organic fraction was washed with brine. After it was dried over sodium sulfate, the solution was concentrated under reduced pressure. Chromatography on silica gel with a gradient of methylene chloride: methanol (100:0 to 95:5) gave a white solid.

***rac*-3-Hexadecanamido-2-octadecoxy-1-propanol.** Yield, 84%; R_f = 0.35 in methylene chloride:methanol = 100:2. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.89 (m, 6H), 1.27 (broad, 54H), 1.45−1.70 (m, 4H), 2.23 (t, 2H), 3.20−3.80 (m, 7H), 5.77 (t, 1H, J = 5.7 Hz). MS (FAB): m/z 582.2 (M+1).

3-Alkylamido-2-alkoxypropylphosphocholines (2). Method D. To a dry ice-bath-cooled solution of 3-alkylamido-2-alkoxy-1-propanol (0.17 mmol) and trimethylamine (5.1 mmol) in 10 mL of anhydrous acetonitrile was added 2-chloro-2-oxo-1,3,2-dioxophospholane (0.25 mmol). The glass pressure tube was sealed, and the reaction mixture was stirred at 65 °C for 16 h. Then, the tube was cooled on dry ice and broken. After it was warmed up to room temperature, methanol was added to dissolve the precipitate. The solvent was removed, and the product was purified by silica gel chromatography using a gradient of methylene chloride:methanol (5:1 to 5:3).

Method E. To a solution of 3-alkylamido-2-alkoxy-1-propanol (7.8 mmol) in anhydrous diethyl ether/THF (2:1, 225 mL) at 0 °C was added dry pyridine (10 mL) followed by 2-bromoethyl dichlorophosphate (3.3 mL, 21 mmol). The reaction mixture was warmed to room temperature and heated at a gentle reflux for 4 h. After it was cooled in an ice-bath, 6 mL of water was added dropwise and stirring was continued for another hour. The solvent was removed on a rotary evaporator, the residue was taken up in 100 mL of chloroform/methanol (2:1, v/v), and the solution was extracted with 20 mL of water while the pH of the organic phase was kept at 5 by addition of 2 N HCl. The aqueous phase was re-extracted with two portions of chloroform/methanol (2:1, v/v, 50 mL), and the

combined organic phases were dried over sodium sulfate and concentrated. The residue was purified on silica gel column with a gradient of chloroform:methanol (100:0 to 3:1), giving colorless oil or white solid. In some cases, product was used in the next reaction without thorough purification and characterization.

To a mixture of 3-alkylamido-2-alkoxy-1-propyl-2′-bromoethyl phosphate (5.7 mmol) in chloroform:2-propanol:DMF (3:5:5, 195 mL) was added trimethylamine (40% aqueous solution, 41 mL). The solution was heated to 65 °C for 5 h and then allowed to be cooled to room temperature before silver carbonate (196 mg) was added. Heat was reapplied for 1 h. The mixture was cooled and filtered. The solvent was removed in vacuo, and the residue was purified on silica gel column using chloroform:methanol:NH_4OH (75:25:5).

***rac*-3-Hexadecanamido-2-octadecoxypropylphosphocholine (42).** Yield, 46%; R_f = 0.35 in chloroform:methanol:ammonium hydroxide = 75:25:5. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.85 (m, 6H), 1.27 (m, 54H), 1.45−1.70 (m, 4H), 2.30 (t, 2H, J = 6.5 Hz), 3.33 (s, 9H), 3.30−3.60 (m, 6H), 3.60−4.00 (m, 3H), 4.35 (m, 2H). MS (FAB): m/z 747.6 (M+1). HRMS (FAB) calcd for $C_{42}H_{88}N_2O_6P$, 747.6380; found, 747.6324.

Physicochemical and Biological Evaluation. Caco-2 cells were purchased from American Type Culture Collection (Rockville, MD). Eagle's Minimum Essential Medium (EMEM), 0.25% trypsin/0.02% ethylenediaminetetraacetic acid-tetrasodium salt (EDTA-4Na), and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY). Nonessential amino acids (NEAA), Hank's balanced salt solution (HBSS), antibiotic antimycotic solution, MTT, SDS, hexadecylphosphocholine, DPH, and TMA-DPH were purchased from Sigma (St. Louis, MO). Dodecylphosphocholine was purchased from Avanti Polar Lipids Inc. (Alabaster, AL). *N*-(2-Hydroxyethyl) piperazine-*N*′-2-ethanesulfonic acid (HEPES) was obtained through Tissue Culture Facilities (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 was purchased from DuPont NEN (Boston, MA). The stock solutions of 3-alkylamido-2-alkoxypropylphosphocholines (50 mM) were obtained by dissolving the phospholipids in HBSS/ethanol (50:50, v/v) and were stored at −20 °C.

Caco-2 and MDCK Cell Cultures. Caco-2 cells were grown in EMEM containing 10% FBS, 1% l-glutamine, 1% NEAA, and antibiotics (100 U/mL of penicillin, 100 mg/mL of streptomycin, and 0.25 g/mL of amphotericin B) 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 1.0 × 10^5 cells/mL on to porous polycarbonate filter membranes with a pore size of 3.0 μm and a surface area of 1.0 cm^2. Cells from passage number 45−55 were used in all of the studies. Media were changed every 2 days after seeding until late confluence (20−22 days). Just before the experiments, the culture medium was replaced with HBSS buffer that contained 1 × HBSS, 25 mM HEPES, and 25 mM glucose at pH 7.4 and incubated for 1 h at 37 °C. The cell monolayers with TEER values in the range of 600−800 Ω·cm^2 were used for the experiments. MDCK cells were passaged and seeded in the same manner as Caco-2 cells, except that MDCK cells were maintained on filter membrane for 4 days to reach confluence. MDCK cells from passage number 65−75 with TEER values in the range of 250−350 Ω·cm^2 were used in all the studies

TEER Measurement (EC_{50} Determination). To measure the effect of the 3-alkylamido-2-alkoxypropylphosphocholines on the TEER values of cell monolayers, the phospholipids were dissolved in 0.5 mL of HBSS at various concentrations and applied to the apical side of the cell monolayers for 20 min at room temperature. The TEER value was measured with an Epithelial Tissue Voltohmmeter (EVOM, World Precision Instruments, Sarasota, FL) and calculated as Ω·cm^2. The resistance caused by the cell monolayer was determined after subtracting the contribution of the blank filter and HBSS buffer. EC_{50} was calculated as the concentration at which the phospholipid decreases the TEER of cell monolayer by 50% of the control (untreated) value. All measurements were in triplicate and expressed as mean ± SD values.

Measurement of Mannitol Transport Rate (EC_{10x} Determination). All transport studies were performed at 37 °C under sink condition (i.e., the concentration of the transported compound in the receiver compartment was less than 10% of its initial concentration in the donor compartment). Permeability coefficients (P_{app}) were calculated using the following equation: $P_{app} = dQ/(dt \times C_0 \times A)$, where dQ/dt (mol transported/sec) is the flux of the marker compound across the Caco-2 cell monolayer, A (cm^2) represents the diffusional area of the inserts, and C_0 (M) denotes the initial concentration of marker compound in the donor compartment. To calculate the enhancer concentration at which the permeability of mannitol is increased by 10-fold (EC_{10x}), the enhancer solution at concentration range of 0.01−5.0 mM in HBSS (0.5 mL) was added to the apical side of the cells. HBSS (1.5 mL) was added to the basolateral side. After treatment for 20 min, the enhancer solution in the apical compartment was removed and replaced with fresh HBSS after washing the cell monolayer once with HBSS. Following the measurement of TEER, the HBSS on the apical side was replaced with 0.5 mL of HBSS solution containing 0.1 mM [^{3}H]mannitol (0.25 μCi). Samples from the basolateral side were taken at 20, 40, 60, and 90 min. Transport rates were determined by measuring the radioactivity in the basolateral side with a liquid scintillation counter. All measurements were in triplicate and expressed as mean ± SD values.

Cytotoxicity Assay. The cell viability was measured by the MTT assay.[45−47] Approximately 3.0 × 10^3 Caco-2 cells (in 100 μL of cell culture medium) were seeded into each well in a 96 well tissue culture plate (Corning-Costar). The cells were cultured in the same manner as described previously (Caco-2 and MDCK cell cultures) for 96 h. The culture medium was changed once. Just prior to the start of each experiment, the medium was removed from the wells, and 100 μL of the 3-alkylamido-2-alkoxypropylphosphocholine solutions in HBSS was added to each well. After 20 min, 20 μL of MTT solution (5 mg/mL) was added to each well, and the cells were incubated for another 120 min. Then, 100 μL of 10% SDS in 0.02 M HCl/isobutanol (1:1, v/v) solution was added to stop the reaction. The cells without the treatment of any phospholipid were harvested as above and were used as controls. Absorbance was measured at 590 nm (indicative of the formation of the formazan product by mitochondrial dehydrogenase of the viable cells) using a multiwell scanning spectrophotometer (Bio-Rad, Hercules, CA). IC_{50} represents the concentration of the phospholipid that causes 50% cells to be incompetent with respect to mitochondrial dehydrogenase activity. All measurements were in triplicate and expressed as mean ± SD values.

CMC Determination. The aqueous solutions of 3-alkylamido-2-alkoxypropylphosphocholine at appropriate concentration range were prepared. To each 2 mL solution of phosphocholine at various concentrations was added 1 μL stock solution of 10 mM DPH in THF. The solutions were mixed thoroughly and incubated in darkness at room temperature for 30 min before fluorescence was measured by a Perkin-Elmer Spectrofluorometer set at excitation wavelength of 365 nm and emission wavelength of 460 nm. The excitation and emission bandwidths were set at optimal width to achieve highest sensitivity. Linear regression analysis on the sets of points below and above the inflection point of fluorescence intensity formed two straight lines that intersected at the CMC. Mean CMC was calculated from triplicates measurements for each compound.

Membrane Fluidity Measurement. Caco-2 cells cultured on Transwell membranes were treated with 0.2% EDTA in PBS solution for 5 min and trypsinized. The cells were suspended, collected by centrifugation, washed twice with HBSS, and diluted in HBSS to a concentration of 2.0 ×10^5 cells/mL. Caco-2 cells were labeled with DPH by adding 2.5 μL of 1 mM freshly prepared stock solution in THF to 2.5 mL

of cell suspension in HBSS and incubated at room temperature for 30 min before fluorescence measurements were made. Alternatively, Caco-2 cells were labeled with TMA-DPH by adding 2.5 μL of 0.5 mM freshly prepared stock solution in DMF to 2.5 mL of cell suspension in HBSS and incubated at room temperature for 2 min before fluorescence measurements were made. These labeling conditions have been found to allow maximal fluorescent probe incorporation and to allow stable measurements over the experiment period.[57]

Fluorescence anisotropy was measured with an SLM-AMINCO Subnanosecond Lifetime Fluorometer, model 4800c, equipped with a Zenith 158 computer for data analysis as described by Audus et al.[35,36] In brief, photomultiplier tubes were placed to the right and left of the dual chamber sample cell with Glans-Thompson polarizers inserted in emission and excitation beams. A Schott KV-389 filter was inserted into the horizontally polarized emission beam, and an SLM MC320 monochromator set at 430 nm was placed in the vertically polarized emission beam. Fluorescence intensity was first measured with the sample excited at 360 nm with a horizontally polarized beam of light and then measured a second time with the sample excited with a vertically polarized beam of light. Corrected fluorescence anisotropy data were calculated from the relationships: $P = (A/B - 1)/(A/B + 1)$, $r = 2P/3 - P$, where P was the fluorescence polarization, A was the ratio of fluorescence intensities parallel and perpendicular to the plane of vertically polarized excitation light, B was the ratio of fluorescence intensities parallel and perpendicular to the plane of horizontally polarized excitation light, and r was the fluorescence anisotropy. Under these conditions, the effect of light scattering will be negligible. Each sample was measured six times, and the average was calculated. Caco-2 cells were maintained in suspension by stirring, for both labeling and fluorescence experiments, with a magnetic stirrer. Two sample cuvettes containing cell suspension were always used as follows: one received indicated compound and the other, as a control, received an equivalent amount of solvent in which the compound was dissolved.

Determination of ATP-Stimulated PLC Activity in MDCK Cells. The activity of PLC in MDCK cells was determined by an adaptation of a previously published method.[58] MDCK cells were seeded at 400 000 cells/well in a 12 well plate and subsequently cultured for 4 days. MDCK monolayers were then labeled with [^{3}H]myo-inositol (1.6 μCi/well in 0.4 mL of inositol-free media) for 24 h at 37 °C. Assays were initiated on labeled cells that were removed from the incubator by immediately supplementing the cells with 100 μL of 250 mM HEPES (pH 7.3), containing 100 mM LiCl (to inhibit hydrolysis of inositol monophosphate to inositol) with or without the test compound. Subsequently, the cells were incubated for 30 min at 37 °C. Immediately after the termination of this incubation, ATP (final concentration: 100 μM) was added. The cells were then incubated at 37 °C for 15 min to allow accumulation of [^{3}H]inositol phosphates. Incubations were terminated by aspiration of the media and the addition of 1 mL of boiling 10 mM EDTA (pH 8.0). The supernatant was applied to AG1 × 8 formate columns for chromatographic isolation of [^{3}H]inositol phosphates.[59] The amount of [^{3}H]inositol phosphates was measured by liquid scintillation counting in a Packard Tri Carb 4000 Series spectrophotometer. Data from each experiment were normalized to the response observed with 100 μM ATP (without treatment with test compounds) and were reported as the mean ± SD of three experiments performed in triplicate. The $IC_{50(PLC)}$ was determined by the concentration of compound that causes a 50% decrease in ATP-stimulated accumulation of [^{3}H]inositol phosphates.

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Supporting Information Available: Detailed characterization of the final products is provided. This material is available free of charge via the Internet at http://pubs.acs.org

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