Effect of Some Penetration Enhancers on Epithelial Membrane Lipid Domains: Evidence from Fluorescence Spectroscopy Studies

T. Marjukka Turunen,[1,3] Arto Urtti,[2] Petteri Paronen,[2] Kenneth L. Audus,[1] and J. Howard Rytting[1]

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The effect of the penetration enhancers Azone, oleic acid, 1-dodecanol, dodecyl *N,N*-dimethylaminoacetate (DDAA), and dodecyl *N,N*-dimethylaminoisopropionate (DDAIP) on epithelial membrane lipids was examined using human buccal cell membranes as a model for epithelial lipid bilayer. Buccal epithelial cells (BEC) were labeled with 1,6-diphenyl-1,3,5-hexatriene (DPH), 1-(4-(trimethylammonio)phenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), and 8-anilino-1-naphthalene sulphonic acid (ANS) fluorophores to characterize enhancer-induced changes in the hydrophobic core, in the superficial polar head region, and on the exterior surface, respectively, with fluorescence anisotropy and fluorescence lifetimes. All the enhancers studied were found to decrease the BEC membrane lipid packing order in a concentration-dependent and time-dependent manner in the deep bilayer region, as shown by a 37–66% decrease in anisotropy. Oleic acid was also found to disrupt membrane lipids strongly in the polar head region, causing at least a 34% decrease in anisotropy values. Azone and DDAA were shown to alter molecular movement on the surface of the bilayers (24 and 19% decrease in anisotropy, respectively). The results suggest that interaction with membrane lipid domains is an important, but not the only, mode of action for the penetration enhancers studied.

KEY WORDS: penetration enhancement; fluorescence polarization; membrane fluidity; fluorescent probes.

INTRODUCTION

The lamellar intercellular lipid domains of the skin and buccal epithelium have been shown to provide a barrier for drug permeation (1–3) and are targets of penetration enhancer action. Stratum corneum lipid conformational changes and drug flux have been found to be strongly correlated (4). For example, the action of oleic acid as a transdermal penetration enhancer has been related to its lipid fluidizing effect (5,6). Fluorescence polarization is a method commonly used to monitor the degree of molecular packing order in the apolar regions of cell membranes (7–9). The fluorescent probes DPH (1,6-diphenyl-1,3,5-hexatriene), TMA-DPH [1-(4-(trimethylammonio)phenyl)-6-phenyl-1,3,5-hexatriene], and ANS (8-anilino-1-naphthalene sulfonic acid) were used to analyze lipid fluidity to epithelial lipid domains in this study. When embedded in the membrane and excited by polarized light, the probe reorients during the time elapsing between excitation and emission. The orientation of emitted light depends on the degree of molecular reorientation. The steady-state fluorescence anisotropies of DPH and TMA-DPH in biomembranes represent the degree of structural order in the hydrophobic core and the surface of the lipid bilayer, respectively. ANS serves as a marker for molecular movement on the exterior of lipid bilayers. A decrease in the anisotropy signifies an increase in the probe rotational cone angle and, consequently, a reduction in the packing order of the corresponding hydrophobic region. The objective of the present study is to evaluate the distribution and effect of five penetration enhancers [Azone, oleic acid, 1-dodecanol, dodecyl *N,N*-dimethylaminoacetate (DDAA), and dodecyl *N,N*-dimethylaminoisopropionate (DDAIP)] on the epithelial lipid domains. Human buccal epithelial cell membrane was used as a model for epithelial lipid bilayer. Buccal epithelial barrier properties and lipid composition have been shown to resemble closely that of the skin (3,10).

MATERIALS AND METHODS

Reagents

1-Dodecanol and oleic acid purchased from Sigma Inc. (St. Louis, MO). Azone was obtained from Nelson Research (Irvine, CA). DDAA (11) and DDAIP (12) were synthesized in our laboratories. The purity of DDAA and DDAIP was more than 99%. DPH and TMA-DPH were obtained from Molecular Probes (Eugene, OR), and ANS was from Sigma Inc. (St. Louis, MO). All other chemicals were reagent grade and used as received.

Enhancer Preparations

Suspensions of DDAA and DDAIP were prepared in 5% (w/v) ethanol/0.01 *M* phosphate-buffered saline (PBS), pH 7.4. Suspensions of Azone, oleic acid, and 1-dodecanol were prepared in 20% EtOH/PBS to facilitate suspension in PBS.

Buccal Epithelial Cell (BEC) Preparations

BECs were obtained by scraping the buccal mucosa of healthy nonsmoking volunteers, BECs were suspended in 5% EtOH/PBS and passed through a small-bore needle (21 G) several times to obtain a single cell suspension. The cell suspension was washed three times with 5% EtOH/PBS by centrifugation at 200*g* for 5 min. The cells were resuspended in 5% EtOH/PBS, counted in a hemacytometer, and diluted to 2×10^5 cells/mL.

Fluorescence Polarization Studies

BEC suspensions were labeled with DPH by adding 2.5 μL of a 1 m*M* stock of DPH in tetrahydrofuran to 2.5 mL of the cell suspension and incubating for 30 min at room temperature in the absence of light. Alternatively, BEC suspensions were labeled with TMA-DPH by adding 2.5 μL of a 5

[1] Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, Kansas 66045.
[2] Department of Pharmaceutical Technology and A. I. Virtanen Institute, University of Kuopio, Kuopio, Finland.
[3] To whom correspondence should be addressed at Department of Pharmaceutical Technology, University of Kuopio, P.O. Box 1627, SF-70211 Kuopio, Finland

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$\times 10^{-4}$ *M* stock of TMA-DPH in dimethylformamide to 2.5 mL of cell suspension and incubating for 2 min. ANS stock solution (50 m*M*) was prepared in PBS. BECs were labeled by adding 2.5 μL of the stock to 2.5 mL of the cell suspension for 5 min. Immediately after labeling, the BEC suspensions were equilibrated to 37°C in the fluorometer equipped with a temperature-regulated stir cell and used for experiments. The steady-state fluorescence anistropy (*r*) was measured using an SLM-AMINCO 4800 Subnanosecond Lifetime Spectrofluorometer (Champaign, IL) as described previously (13). Experiments were conducted with paired cuvettes each containing 2.5 mL of labeled cells in 5% EtOH/PBS. After the initial *r* was recorded for the untreated BECs, an enhancer suspension (0.05–3.5 m*M*) was added to the cuvette, and the control cuvette received equivalent volumes of the appropriate solvent. The apparent *r* was recorded until no further changes were observed in the anisotropy readings (up to 50 min). All measurements were made at 37°C.

Fluorescence Lifetime Measurements

The fluorescent probe lifetime was measured with an SLM-AMINCO 4800 Spectrofluorometer by the phase sift demodulation method using a modulation frequency of 30 MHz as described earlier (13). Dimethyl-POPOP with a known lifetime of 1.45 nsec was used as a reference. Otherwise, experiments were conducted in a manner similar to that for the anisotropy studies.

Light Scattering Measurements

The influence of the turbidity of the cell suspension on the fluorescence measurements was determined in the presence and absence of the enhancers at two wavelengths, 540 and 350 nm (14). The same wavelength was used for excitation and emission, with cutoff filters of KV470 and 240–330 for 540 and 350 nm, respectively. An aliquot of the enhancer suspension of the highest concentration used in the experiments was introduced into the unlabeled cell suspension. The control cuvette received equivalent volumes of the pure solvent. Changes in the fluorescence emission was monitored with time.

Esterase Activity in BECs

DDAIP and DDAA are subject to degradation, and therefore inactivation, by esterases; thus levels in these cells were determined to assess their potential in limiting effects of these enhancers on the tissue. Esterase activity in BEC was determined qualitatively using a nonspecific α-naphtyl acetate (NAE) assay (Sigma, St. Louis, MO). Heat-treated BECs without any enzymatic activity were used as controls. BEC suspensions in 5% EtOH/PBS were washed twice with deionized water by centrifugation and incubated with NAE for 30 min at 37°C protected from light, rewashed, and counterstained for 2 min in hematoxylin solution, Gill No. 3. α-NAE activity was evaluated microscopically after resuspension of the BECs in deionized water.

Data Treatment

The significance of the differences between the groups was tested statistically using repeated-measures one-factor ANOVA. Thereafter, Scheffe's *F* test was used to compare each of the enhancer groups to the control group.

RESULTS

Fluorescence Anistropy Studies

Figure 1 illustrates the time-dependent effects of the enhancers (1 m*M*) on the DPH fluorescence anistropy in buccal epithelial cells. Oleic acid exhibits the strongest decrease in anisotropy and equilibrium is attained in less than 10 min, most likely reflecting the favorable solubility of the enhancer in that DPH-labeled domain relative to the other enhancers. For the other enhancers, interaction with DPH-labeled BEC membranes takes longer: about 30 min for DDAA and up to 40–50 min for Azone, 1-dodecanol, and DDAIP. Addition of the solvent (controls) did not alter DPH anisotropy with time.

Figure 2 illustrates the dose-dependent effects of the enhancers of the DPH fluorescence anisotropy. With increasing concentrations of the enhancers, there is a corresponding decrease in the fluorescence anisotropy of DPH. Additions of equal volumes of pure solvent did not alter DPH anisotropy. There was no significant difference between the effects of the two solvent formulations used (5% EtOH/PBS and 20% EtOH/PBS). Reproducible enhancer effects on the DPH anisotropy begin at a concentration of about 0.2 m*M*, except with oleic acid, which caused a significant decrease in the DPH anistropy at a concentration of about 0.1 m*M*.

The possible interference of the turbidity of the cell suspension with the fluorescence measurements was determined by studying light scattering characteristics of the cell suspension at 540 nm. An enhancer suspension of the highest concentration used in the DPH anisotropy studies (2.0–3.3 m*M*) was introduced into the unlabeled BEC suspension. The control group received equal volumes of the solvent. Fluorescence of the mixed suspension increased strongly in all cases (Fig. 3), which indicates an increased turbidity of the cell suspension caused by the addition of concentrated enhancer suspensions. The result suggests that the data in Fig. 2 may reflect micelle formation and/or possible disintegration of cell suspensions when high concentrations of enhancers are used, rather than a saturation of enhancer action. Similar results also were obtained at 350 nm (data not shown). Therefore, a lower enhancer concentration range ($\leq$1 m*M*), where concentration and response showed a linear relationship, was chosen for further studies.

Oleic acid exhibits a strong effect also on the TMA-DPH fluorescence anisotropy (Fig. 4). Anisotropy decreases with increasing oleic acid concentration until a limiting concentration value of about 0.7 m*M*, when the maximum decrease in anisotropy is attained. At a concentration of about 1 m*M*, the TMA-DPH anisotropy was slightly reduced by 1-dodecanol, DDAA, and Azone as well, whereas additions of DDAIP or pure solvent did not cause any effect. Azone and DDAA were shown to decrease the ANS fluorescence anisotropy in a concentration-dependent manner (Fig. 5), whereas the other enhancers or pure solvent were shown not to produce a consistent effect.

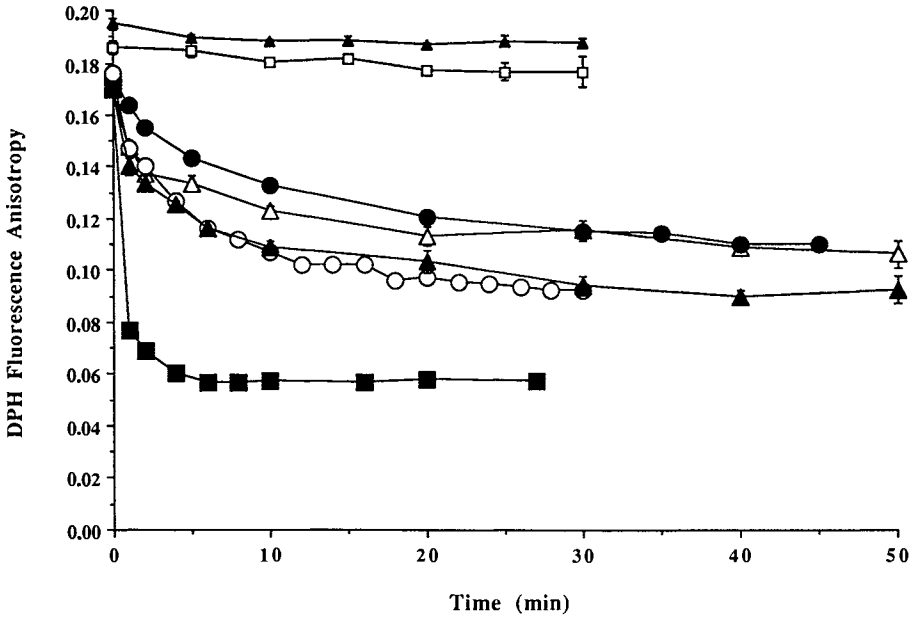

Fig. 1. Time course of enhancer effects on the DPH fluorescence anisotropy in BEC at 37°C. A 1 m*M* enhancer suspension was added to the DPH-labeled BEC suspension and the fluorescence anisotropy recorded until no change was observed. The initial anisotropy values were in the range of 0.170–0.176. Mean ± SD; $n = 6$. Azone (▲); oleic acid (■); 1-dodecanol (△); DDAA (○); DDAIP (●); control, 5% EtOH/PBS (▲); control, 20% EtOH/PBS (□).

Fluorescence Lifetime Studies

The fluorescence lifetime of a probe embedded in the membrane is sensitive to the changes in its microenvironment (15), and thus it is used to interpret data from anisotropy measurements properly. For example, quenching of the probe fluorescence emission would result in an appreciable decrease (i.e., 50% or more) (16) in the probe lifetime and would also significantly affect the anisotropy values recorded. In the present study, an anisotropy change of about 20% or more was tested for the fluorescence lifetime to reveal possible probe–enhancer interactions. Table I summarizes the results from the anisotropy and lifetime measurements at a 1 m*M* enhancer concentration. The DPH fluorescence lifetime decreased in the presence of 1 m*M* 1-dodecanol, DDAA, and Azone. Oleic acid was shown to increase

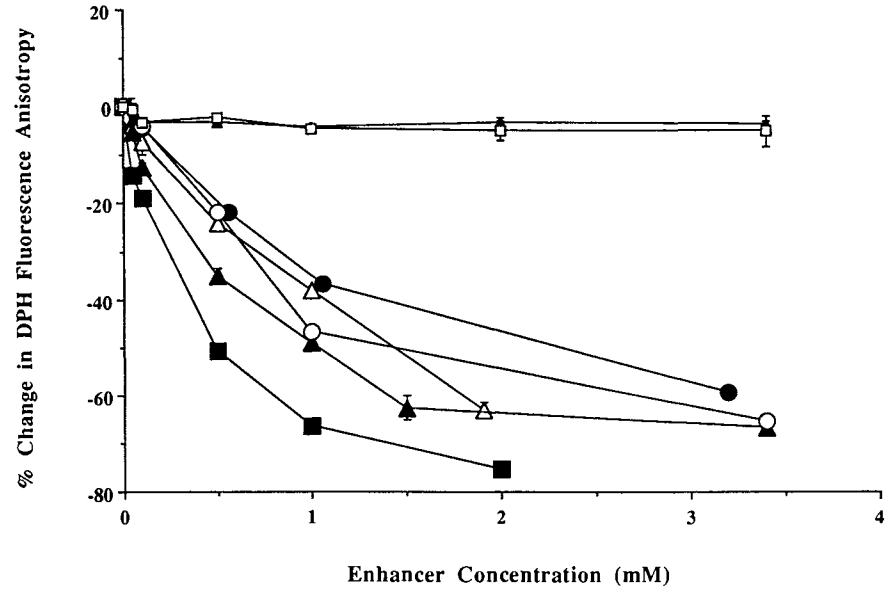

Fig. 2. The effect of enhancers on the DPH fluorescence anisotropy in BEC at 37°C. The initial anisotropy values were in the range of 0.161–0.182. Mean ± SD; $n = 6$. Azone (▲); oleic acid (■); 1-dodecanol (△); DDAA (○); DDAIP (●); control, 5% EtOH/PBS (▲); control, 20% EtOH/PBS (□).

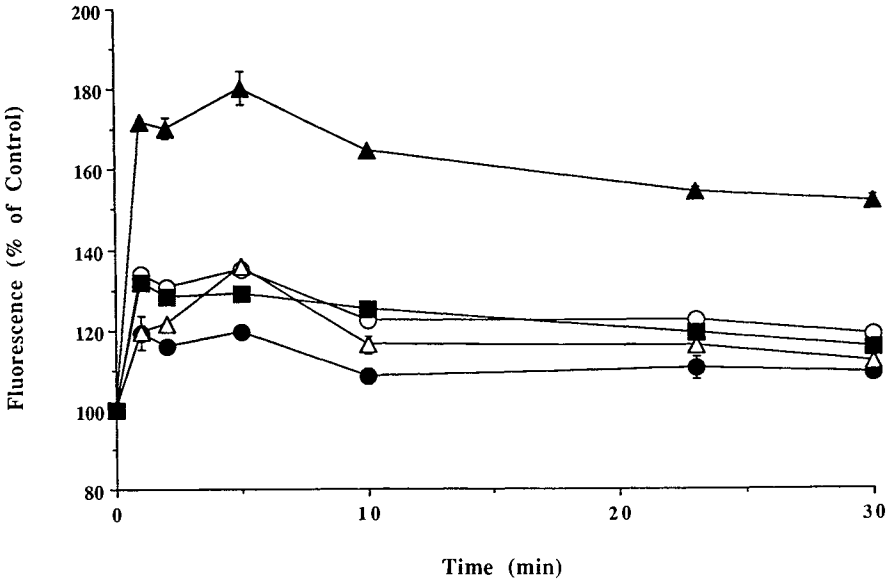

Fig. 3. Light scattering effects of enhancers in the BEC suspension at 37°C (540 nm). An aliquot of a concentrated (2.0–3.3 m*M*) enhancer suspension was introduced to the BEC suspension and changes in fluorescence were observed. Mean ± SD; $n = 6$. Azone (▲); oleic acid (■); 1-dodecanol (△); DDAA (○); DDAIP (●).

the DPH fluorescence lifetime slightly, whereas DDAIP did not have any effect. ANS fluorescence lifetime was slightly increased by 1 m*M* Azone. However, changes of less than 50% in the DPH and ANS lifetimes were interpreted as not being an indication of a significant quenching of the probe fluorescent emission by the enhancers (16). Oleic acid, in contrast, decreased the TMA-DPH fluorescence lifetime by 63%, which may indicate its direct interaction with the TMA-DPH molecule or, perhaps, changes in the probe partitioning between the lipid phases.

Esterase Activity in BECs

Esterase activity present in BEC suspensions was determined with the aid of a commercial nonspecific esterase enzyme assay kit (α-NAE). BECs were incubated with α-NA in the presence of freshly formed diazonium salt. Enzymatic hydrolysis of ester linkages liberates free naphthol compounds. These couple with the diazonium salt, forming highly colored deposits at sites of enzyme activity. Heat treatment was used to denature the enzyme proteins in the

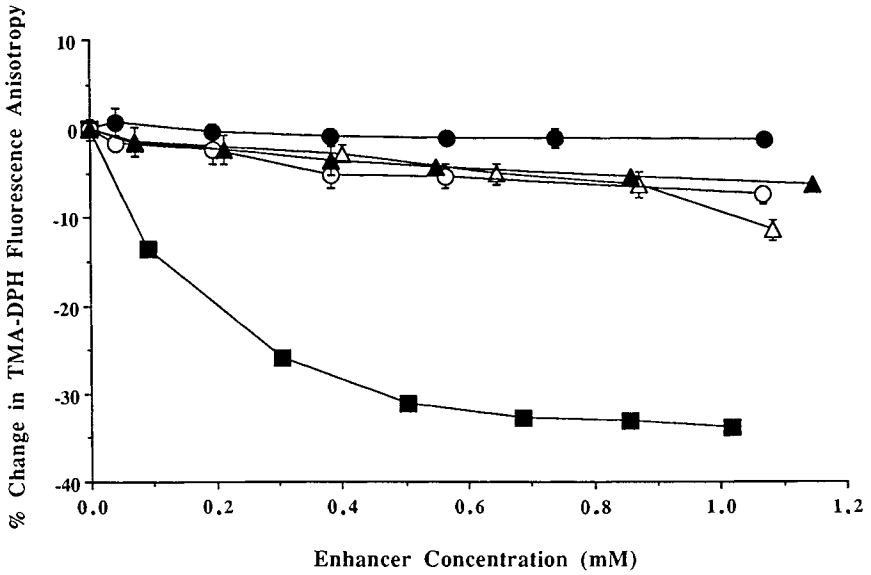

Fig. 4. The effect of enhancers on the TMA-DPH fluorescence anisotropy in BEC at 37°C. The initial anisotropy values were in the range of 0.280–0.286. Mean ± SD; $n = 6$. Azone (▲); oleic acid (■); 1-dodecanol (△); DDAA (○); DDAIP (●).

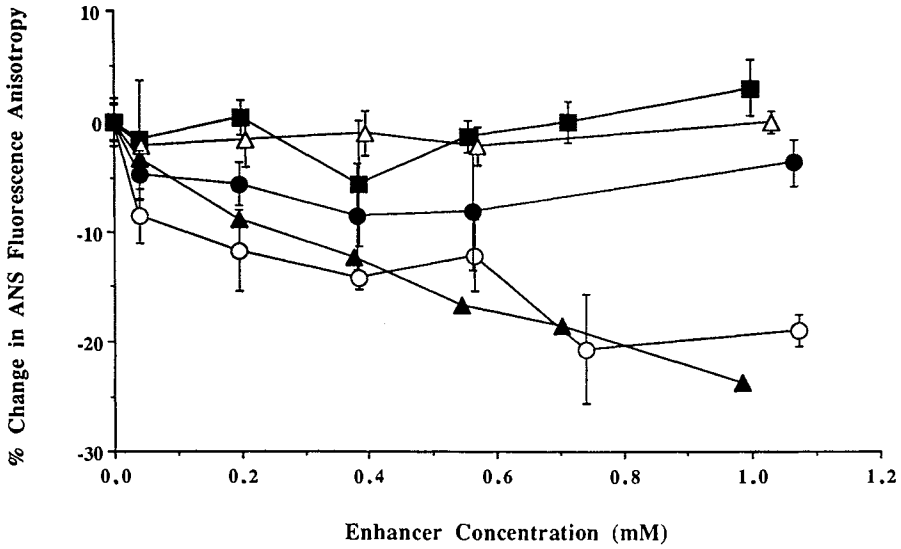

Fig. 5. The effect of enhancers on the ANS fluorescence anisotropy in BEC at 37°C. The initial anisotropy values were in the range of 0.283–0.300. Mean ± SD; $n = 6$. Azone (▲); oleic acid (■); 1-dodecanol (△); DDAA (○); DDAIP (●).

control cells, and after staining, no colored deposits were detected. In untreated cells, however, a few black granules were found, indicating the presence of esterases.

DISCUSSION

This study compares the effects on the epithelial lipid domains due to different penetration enhancers. In the fluorescence polarization method, the behavior of the probes provides information on their immediate surroundings (17). By the use of a set of probes located in different lipid regions, membrane fluidity alterations can be characterized and reference to the enhancer action addressed. The apolar DPH is assumed to partition into the deep regions of the lipid bilayer, although its precise location is uncertain (7). TMA-DPH, a cationic derivative of DPH, would be anchored to the polar head-groups in the bilayer, the DPH moiety being aligned with the lipid acyl chains (9). ANS serves as a marker for the molecular movement on the exterior surface of the lipid bilayer, binding to membranes being strongly dependent on the surface charge of the membrane (18). The hypothesis that TMA-DPH is located in more ordered regions than DPH is supported by the observation that TMA-DPH usually exhibits higher anisotropy values than DPH (17). Similar results were obtained in the present study (Figs. 1 and 4). Moreover, ANS anisotropy values were close to those of TMA-DPH or slightly higher (Fig. 5), suggesting that the surface and the polar head-group region of the buccal epithelial cell membrane are more ordered than the deep acyl-chain region. It also has been reported that porcine stratum corneum lipids nearest to the polar end of long-chain molecules are highly ordered (19). Our results are in agreement with this observation and indicate that the lipid domains in buccal epithelial cells structurally are similar to the intercellular lipid domains of stratum corneum. Fluorescence anisotropy has been used in a number of studies to monitor changes in the structural order of the lipid domains in biomembranes (7,8,20) and artificial membranes (9). The decrease in fluorescence anisotropy may be appropriately interpreted as a decrease in the structural order of the lipid

Table I. Fluorescence Anisotropy Change (%) and Fluorescence Lifetime Change (%) in Buccal Epithelial Cell Membranes at a 1.0 m*M* Enhancer Concentration: Mean (SD), $n = 6$ (37°C)

	Change (%) in					
	DPH		TMA-DPH		ANS	
Enhancer	Anisotropy	Lifetime	Anisotropy	Lifetime	Anisotropy	Lifetime
Control (5% EtOH–PBS)	−3 (0.2)		−1 (0.2)		+1 (1)	
DDAIP (″)	−37 (1)*	−2 (3)	−1 (1)		−4 (2)*	
DDAA (″)	−47 (1)*	−15 (5)	−7 (1)*		−19 (2)*	
Control (20% EtOH–PBS)	−5 (1)		+1 (0.3)		+8 (2)	
Azone (″)	−49 (1)*	−21 (6)	−6 (0.1)*		−24 (0.4)*	+16 (12)
Oleic acid (″)	−67 (0.3)*	+13 (4)	−34 (0.2)*	−63 (1)	+3 (3)*	
1-Dodecanol (″)	−38 (0.4)*	−12 (4)	−11 (1)*		No change*	

* Significantly different from the control group at the 99% confidence level (Scheffe's *F* test).

microenvironment of the probe. All the enhancers studied were found to decrease extensively the lipid packing order in the deep lipid bilayer region of BEC membranes (Table I), indicated by the decrease in the DPH anisotropy. All the enhancers, except DDAIP, slightly decreased the lipid order in the superficial hydrophobic region of the membranes, indicated by the TMA-DPH fluorescence anisotropy decrease. Azone and DDAA were also found to alter molecular movement on the exterior surface of BEC membranes, indicated by changes in the ANS fluorescence anisotropy.

None of the enhancers, except oleic acid, produced a significant effect on the fluorescence lifetimes. The results from anisotropy studies, thus, can be interpreted as enhancer-induced perturbations of the structural order of the corresponding lipid microenvironments of the probe molecules in BECs. Oleic acid, however, strongly decreased the TMA-DPH fluorescence lifetime, which may be an indication of its direct interaction with the TMA-DPH molecule and quenching of the fluorescence emission to some extent. Changes in the fluorescence lifetime affect the fluorescence anisotropy according to the Perrin equation (5):

$$r = \frac{r_0}{1 + (\tau/\phi)}$$

where r is the fluorescence anisotropy, r_0 is the maximal theoretical fluorescence anisotropy value in the absence of any rotational motion, τ is the fluorescence lifetime, and ϕ is the rotational correlation time. In a fluid environment, $\tau \gg \phi$ (15), and thus, a significant decrease in τ would result in an increase in r. Our results, however, show decreased TMA-DPH anisotropy caused by oleic acid, which together with the results from lifetime studies, reflects possible changes in the rotational correlation time (ϕ). A decrease in ϕ, consequently, indicates less hindrance in the rotational movement of the probe, suggesting a real decrease in the lipid packing order in the polar head region of BEC membranes caused by oleic acid. The anisotropy values may be offset by the direct interaction between oleic acid and the TMA-DPH molecule, and thus the absolute magnitude of the fluidizing effect of oleic acid would be greater than observed. A change in the fluorescence lifetime may also indicate alterations in partitioning of the probe (15), i.e., from the inherent membrane lipid phase to the oleic acid phase.

The ethanol/PBS solvents caused slight changes in the DPH, TMA-DPH, and ANS fluorescence anisotropies, the effect being stronger with increasing ethanol content (Table I). The results could be attributed to ethanol effects on BEC membranes. Ethanol has been shown to extract lipid from human stratum corneum lipid domains (21). Extraction of lipid could result in reorganization of the lipid domains, which may be responsible for the decreased DPH anisotropy with increasing ethanol concentration observed in our study. Ethanol has also been suggested to interact with the keratinized protein in human stratum corneum, causing swelling and secondary conformational alterations (22). A similar mechanism may contribute to increased packing order within the superficial membrane domains, as indicated by slightly increased TMA-DPH and ANS anisotropies in the present study.

A relationship between stratum corneum lipid fluidity and skin permeability has been established *in vitro* (4) and *in vivo* (23). Moreover, drug absorption enhancing activity and membrane lipid perturbing effects have been shown to be interrelated in biomembranes (20) and in porcine stratum corneum (6). In the present study, all the enhancers examined were shown to induce lipid disorder in human buccal epithelial cell membranes. Oleic acid strongly reduced the lipid packing in both the hydrophobic core and the polar head-group region of the bilayer. The result is in excellent agreement with the observations reported earlier (5). Azone has been found to increase lipid fluidity (24) and suggested to alter the stratum corneum keratin structure (25), which in turn may result in reorganization in lipid domains. Our results show Azone effects in all membrane regions, again in agreement with the literature. The enhancement effects of DDAA and Azone have been reported to be close to each other for the permeation of several drugs (26,27). Interestingly, DDAA was shown to act on the lipid domains in BECs in a manner similar to that of Azone in our study. DDAA and DDAIP have been designed to be biodegradable in the presence of esterases, dodecanol being one of the degradation products. Consequently, esterases found in the buccal epithelial cells may have some effect on the activity of DDAA and DDAIP, but the absolute magnitude of enzyme activity and true biodegradability of these compounds are difficult to estimate based on our qualitative assay. The actions of DDAA and 1-dodecanol on the BEC membrane lipids seem to follow a similar pattern, with the parent compound having a stronger effect on the membrane surface domains. The lipid distribution of DDAIP, a derivative of DDAA with an additional methyl group, however, was found to be surprisingly different from that of DDAA and 1-dodecanol. In fact, the effect of DDAIP on BEC lipids was shown to be the least of all the enhancers studied. On the other hand, DDAIP has been reported to be a more effective penetration enhancer than DDAA, Azone, and 1-dodecanol for several types of drugs (12,27). It seems apparent that other mechanisms of action, for example, interactions with membrane keratin or with drugs, may contribute significantly to the effectiveness of DDAIP.

This study demonstrates the utility of the fluorescence polarization technique in examining epithelial membrane lipid domains and changes therein. Several penetration enhancers are shown to disrupt lipid packing significantly in epithelial cell membranes, indicating interactions with membrane lipids as their important mode of action. It is also suggested that interactions with other membrane components (e.g., proteins) or direct interaction with drugs may contribute to the action of some enhancers.

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