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Mechanisms of action of some penetration enhancers in the cornea: Laser scanning confocal microscopic and electrophysiology studies

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Summary

The present study investigates the effects of various penetration enhancers including EDTA, digitonin, deoxycholic acid, and a cytoskeleton-active agent, cytochalasin B, on the structure and electrophysiological properties of the rabbit cornea. Using confocal fluorescence microscopy, with the aid of a specific membrane probe (3,3'-dioctadecyloxacarbocyanine perchlorate), and a viability polar tracer (propidium iodide), structural changes and cell death as a result of the penetration enhancers can be detected. In addition to its great sensitivity, the technique provides direct observation, through optical sectioning, of the interior structures of living tissues without the usual artifacts associated with tissue preparation which is required in conventional microscopy. In conjunction with the microscopic method, an electrical method based on membrane resistance and capacitance measurements was used to provide quantitative assessment of the promoting effect of the enhancers. Since electrical resistance is a measure of charge flow and ionic membrane permeability, it can be used to indicate changes in dimension of the aqueous transport pathway, i.e., the tight junction and the intercellular space, as well as the degree of penetrability of the charged species. On the other hand, membrane capacitance reflects the ability of the membrane dielectric to store charges, and thus, it can be used to detect structural damage to the membrane surface. Results obtained from this study indicate that all enhancers significantly increase membrane permeability depending on the concentration and exposure time. However, with the exception of the cytoskeleton-active agent, all enhancers also cause severe membrane damage. Mechanistically, this agent acts specifically on the tight junction proteins and consequently increases intercellular permeability. The chelator, EDTA, acts not only on cell junctions by interfering with calcium ions and altering intercellular integrity, but also disrupts the plasma membrane. Digitonin and bile salt interact with membrane lipid structures and disrupt their organization.

Introduction

Most biological epithelia are relatively impermeable to macromolecules including peptides and proteins. This represents one of the most challenging problems for non-parenteral use of these compounds as potential therapeutic agents. Several attempts have been made over the years to increase membrane permeability to peptides, most notably, through the use of penetration enhancers. Although some of these enhancers seem to be effective, they normally create some undesirable effects associated with tissue irritation and damage. Thus, the real application of these compounds has

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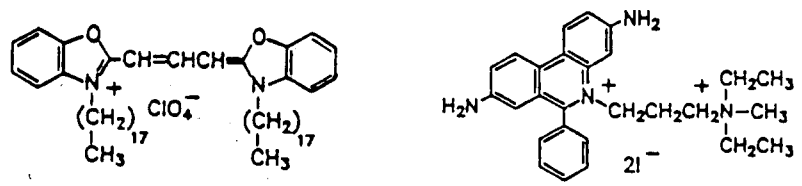
thus far been limited. The purpose of the present study is to investigate the promoting mechanisms of various penetration enhancers in the cornea as well as to develop methods for evaluating tissue damage and viability. In addition, the relative efficacy and toxicity of these enhancers are compared.

To study the mechanisms of enhancers, a sensitive confocal fluorescence microscopic method and a quantitative electrical approach have been used. The basis and application of the two methods to study transport processes in the cornea have been previously reported (Rojanasakul and Robinson, 1990; Rojanasakul et al., 1990). Other comprehensive reviews on the subject have also been described (White et al., 1987; Fine et al., 1988; Shuman et al., 1989). In addition to allowing direct observation, the confocal method offers several advantages over conventional microscopy including (a) improved resolution, contrast, and rejection of out-of-focus interference, (b) by optical sectioning through the interior structures of the tissues, serial images of deeper layers of a thick specimen, that are completely obscured in conventional imaging, can be obtained, and most importantly, (c) it gives the opportunity to study the living tissue without interfering artifacts caused by tissue processing, i.e., chemical fixation, dehydration, embedding, and sectioning, that is normally required in conventional microscopy.

To study the effect of penetration enhancers on membrane structures, two specific fluorescent probes namely 3,3′-dioctadecyloxacarbocyanide perchlorate (DiO), a lipophilic membrane probe, and propidium iodide (PI), a non-permeating polar tracer, were used. The chemical structures of the two compounds are shown in Fig. 1. The lipophilic DiO, due to its membrane specificity, is used to provide a general view of the detailed structures of the plasma membrane. This compound has been shown to possess excellent emission intensity, stability, and low cell toxicity, and thus has been widely used to study membrane-related phenomena in many living systems (Honig and Hume, 1986; Godement et al., 1987; Haugland, 1989). The hydrophilic PI is normally excluded from cells. However, if the cell membrane is disrupted, the probe can enter the cell and bind specifically to the cell nucleus (Haugland, 1989). Upon binding, its fluorescence emission is strongly enhanced and thus intense nuclear fluorescence indicates membrane damage and cell death (Sasaki et al., 1987). The use of the second probe allows a more sensitive detection of membrane damage since detection through direct observation of the membrane probe is limited to resolution of the microscope, i.e., ≈ 0.2 μm. In addition, since DiO and PI have a similar absorption spectrum ($\lambda_{max} \approx$ 490 nm) but exhibit widely separated emission peaks ($\lambda_{max} \approx 639$ and 507 nm, respectively), they can be both excited by argon laser (the light source used in the confocal system) and simultaneously detected.

In addition to microscopic observation, the promoting mechanisms and the effectiveness of the enhancers were investigated by an electrical method. The use of the electrical method to study the effect of drugs in the cornea has been reported previously (Klyce and Wong, 1977; Wolosin, 1988). In the present study, measurements based



3,3'-Dioctadecyloxacarbocyanide Perchlorate
(DiO, MW = 880)

Propidium Iodide
(PI, MW = 668)

Fig. 1. Chemical structures of 3,3′-dioctadecyloxacarbocyanide perchlorate and propidium iodide.

on membrane electrical resistance and capacitance were conducted to provide quantitative assessment of membrane permeability and integrity.

Materials and Methods

Male albino rabbits, New Zealand strain, (New Franken, WI) weighing between 2.5 and 3.0 kg were used. The rabbits were fed a regular diet with no restrictions on the amount of food or water consumed. Lighting was maintained in the caging facilities for 24 h a day to provide a constant experimental environment. 3,3'-dioctadecyloxacarbocyanide perchlorate and propidium iodide were obtained from Molecular Probes Inc. (Eugene, OR) and Na_2EDTA, digitonin, sodium deoxycholate, and cytochalasin B were obtained from Sigma (St. Louis, MO). All chemicals were used as received. Distilled deionized water was used in preparation of the buffer solutions.

Confocal laser scanning microscopy

The MRC-500/Lasersharp fluoroscan confocal system (Biorad, Cambridge, MA) was used. The system consists of a computer controlled laser scanner assembly which attaches to the Nikon Optiphot microscope. An argon ion laser (Ion Laser Technology Inc., Salt Lake City, UT) operating at 488 nm wavelength was used as the excitation source. The two fluorescence signals (green for DiO and red for PI) were detected simultaneously with a dual-channel photomultiplier detection system. The images were assembled in an integral image processor and displayed on a digital video monitor, having a resolution of 768 × 512 pixels. Corneal tissues, taken after specific treatments with or without enhancers were directly mounted, epithelial side up on a glass slide, using a non-fluorescing mounting medium containing glycerol, polyvinyl alcohol and buffer (Polysciences Inc., Warrington, PA). The mounted tissues were then examined microscopically.

In vitro perfusion study

The procedure for isolation and mounting of the cornea was previously described (Rojanasakul and Robinson, 1990). After the cornea was securely mounted, 7 ml of glutathione-bicarbonated Ringer's solution, pH 7.4, was first added to the receptor cell and an equal volume of the same buffer solution containing specific penetration enhancers was subsequently added to the donor cell. The exact concentrations of the enhancers are indicated in the Result section. The experiments were conducted at 37°C for a period of 3 h. Aeration with a mixture of 95% O_2 and 5% CO_2 was also provided. In the confocal study, donor solutions were also spiked with DiO and PI, both having concentrations of 1.0 μg/ml. The addition of the probes to the solutions was found to have no interfering effect on the action of the enhancers as indicated by the similar results obtained when the probes were applied either simultaneously or separately, i.e., 3 h incubation with the enhancers followed by 1 h incubation with the probes.

Impedance measurements

The procedure for impedance measurements and subsequent determination of membrane resistance and capacitance were described previously (Burnette and Bagniefski, 1988; Rojanasakul and Robinson, 1990). Briefly, the method utilizes a four-electrode (Ag-AgCl) system: two electrodes, located 1 cm from each corneal surface, were used to record potential difference and the other two, located 2 cm from each surface, were used to inject current pulses. Alternating, sinusoidal, currents having the intensity of 1 to 10 $\mu A\ cm^{-2}$ and frequency of 1 and 500 Hz, were generated from a function generator. The low frequency impedance (at 1 Hz) was taken as an index for membrane resistance while the impedance obtained at 500 Hz was used to calculate membrane capacitance. All impedance values were obtained from the ratios of the voltage difference across the potential electrodes and the current passing through the tissue. To obtain the actual corneal impedance, impedance between the two sensing electrodes, determined in the absence of the cornea, was substracted from that obtained in the presence of the cornea. All electrical setups were completed within a few minutes after the application of the bathing solutions and the measurements were initiated ≈ 5–10 min thereafter.

Results and Discussion

Effect of penetration enhancers on corneal structures

The key to effective and safe use of penetration enhancers to promote peptide absorption relies on an understanding of the basic transport mechanisms of the peptides in the tissues as well as the mechanisms of action of the enhancers. In the cornea, it has been shown that peptides can permeate the membrane through different transport pathways, depending on the physicochemical properties and membrane specificity of the peptides. For example, the highly charged peptide, polylysine penetrates the membrane through the aqueous intercellular pathway, while insulin, due to its specific surface recognition, penetrates the membrane predominantly via endocytosis (Rojanasakul et al., 1990). Thus, by taking advantage of the transport mechanisms and proper selection of enhancers that selectively modify the transport pathways, effective and perhaps safer delivery of peptides may be achieved. Unfortunately, most presently available enhancers act rather nonspecifically and, with few exceptions, their exact mechanisms are not known. Recently, several sophisticated techniques have been utilized to study the mechanisms of action of these compounds. These include (a) differential scanning calorimetry (Barry, 1987), (b) fluorescence polarization (Kurosaki, 1989), (c) freeze-fracture electron microscopy (Miller, 1984), and (d) conductance measurement (Wheatley, 1988). A complement to this work, the present study utilizes a newly developed laser scanning confocal microscopic technique and an electrical method based on impedance measurements to evaluate the promoting mechanisms of various penetration enhancers. The enhancers being investigated here are (a) the chelator, EDTA, (b) non-ionic surfactant, digitonin, (c) bile salt, sodium deoxycholate, and (d) a newly introduced enhancer, the cytoskeleton-active agent, cytochalasin B.

In confocal microscopy, due to its out-of-focus exclusion capability, only signals at the plane of focus are detected. This allows high contrast and low diffuse background images to be obtained. However, in the present study, when simultaneous detection of signals that originate from different focal levels, such as those from the plasma membrane and nucleus of a cell, is to be performed, difficulties sometimes arise. To circumvent this problem, serial images at different depths through the specimen are first produced and stored in the computer memory of the confocal system. Subsequently, these images are collected, superimposed, and simultaneously displayed. The resulting, 3D-like, image contains all signal information existing in the focal range with high clarity and detail. An example of this image representing the corneal epithelial surface is shown in Fig. 2. In this micrograph, 20 equally spaced serial images with 1 μm increment (10 for DiO-membrane labeling and the other 10, at corresponding depths, for PI-nuclear labeling) through the top 2–3 layers of the epithelial surface are presented. For demonstration purposes, the image depicted here represents the epithelial surface which has been subjected to slight damage. This is simply done by incubating the tissue in GBR buffer for a period of 12 h at 37°C, with regular aeration. Previous studies based on electron microscopy (Rojanasakul and Robinson, 1990) have shown that cornea treated by this way maintains its cellular organization with a slight damage occurring in some of the superficial cells and in the intercellular spaces. In the present study, general preservation of the surface integrity with typical presence of light and dark cells, similar to that observed in scanning electron and light-reflected confocal microscopy (Pfister, 1973; Rojanasakul et al., 1990) is observed. Although the plasma membrane of these cells appears normal (green label), the presence of nuclear fluorescence (red label) indicates disruption of the membrane and presumably cell death.

The effect of penetration enhancers on corneal integrity is shown in Figs. 3–7. In this study, only the effect on the superficial epithelial layer is present due to its overwhelming effect on the transport barrier of the cornea. Probe treatment and optical sectioning of the tissue are similar to that described earlier. The incubation period and enhancer concentration are fixed at 3 h and 1 mM, respectively. In some studies, short-time experiments were also conducted, but their results are difficult to interpret and often inconclusive. For

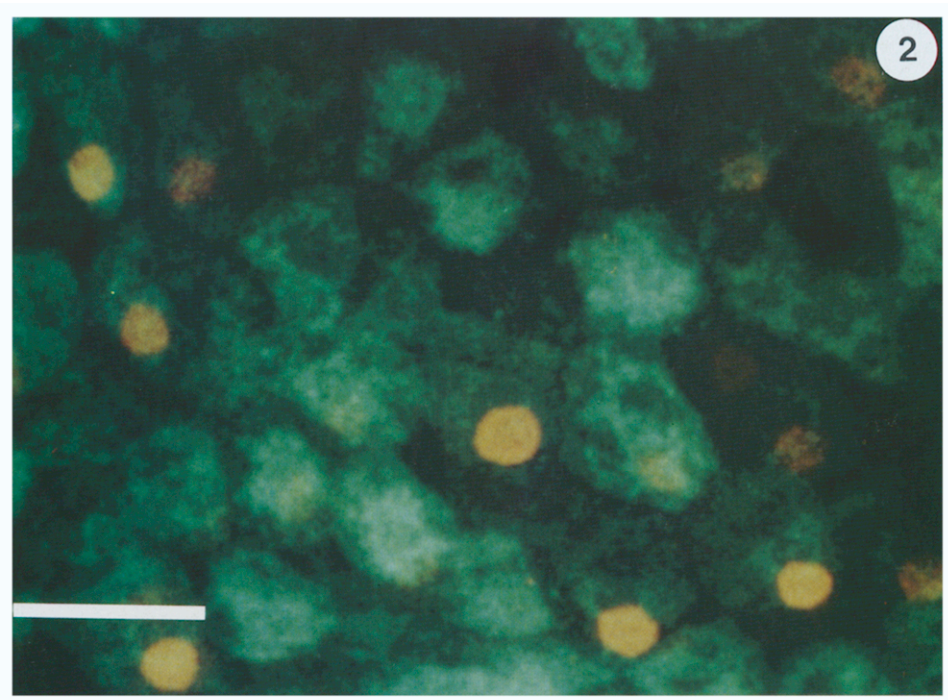


Fig. 2. Confocal fluorescence micrograph of rabbit corneal epithelium. Cell membrane is presented in green (DiO labeling) and nucleus is in red (PI labeling). The presence of nuclear fluorescence indicates disruption of cell membrane. Bar = 50 μm.

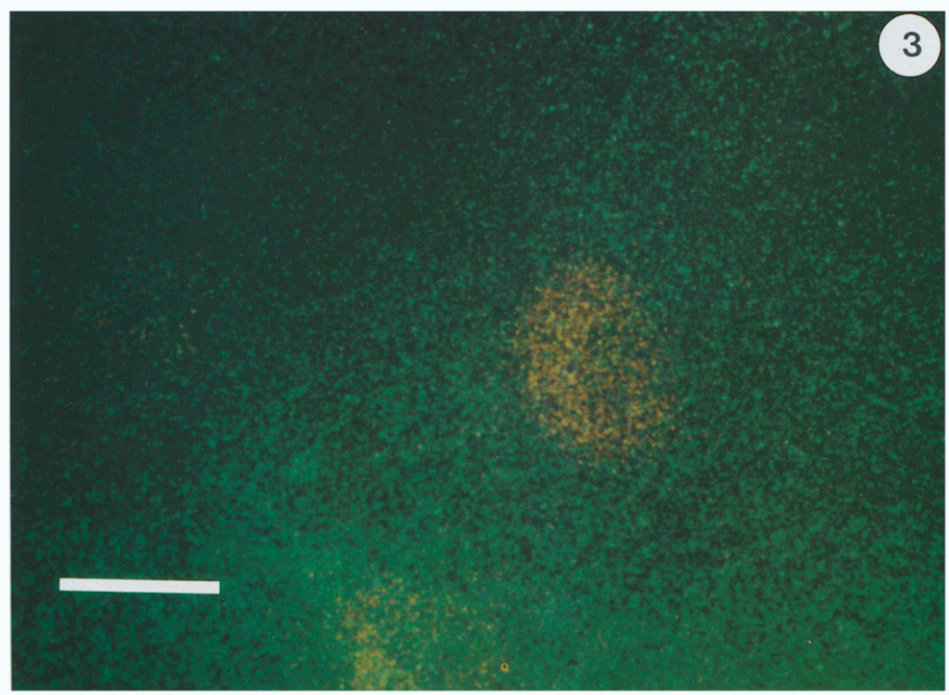


Fig. 3. Surface view of the control corneal epithelium. The control cornea was taken after 3 h in vitro perfusion in GBR buffer. Minimal nuclear fluorescence was observed. Bar = 10 μm.

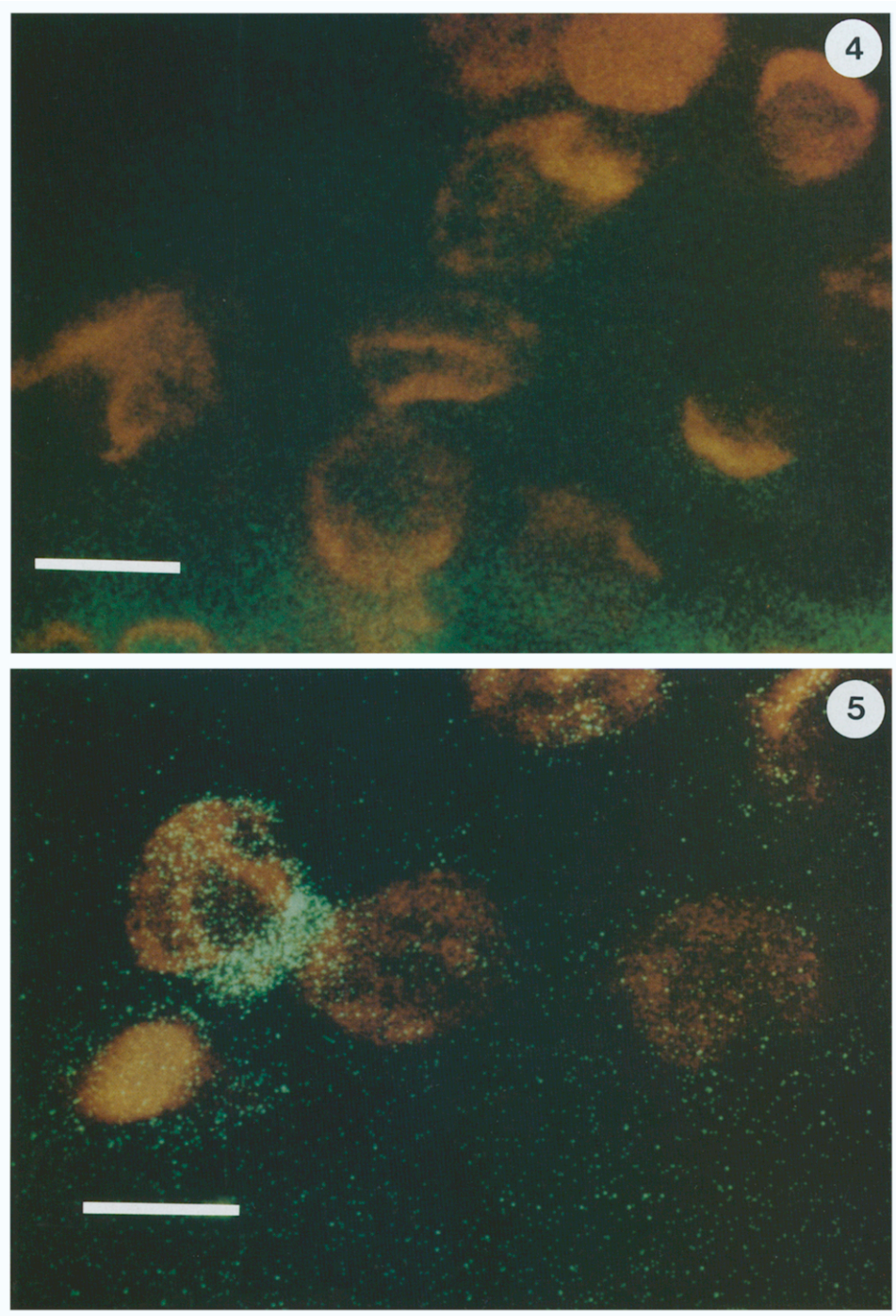
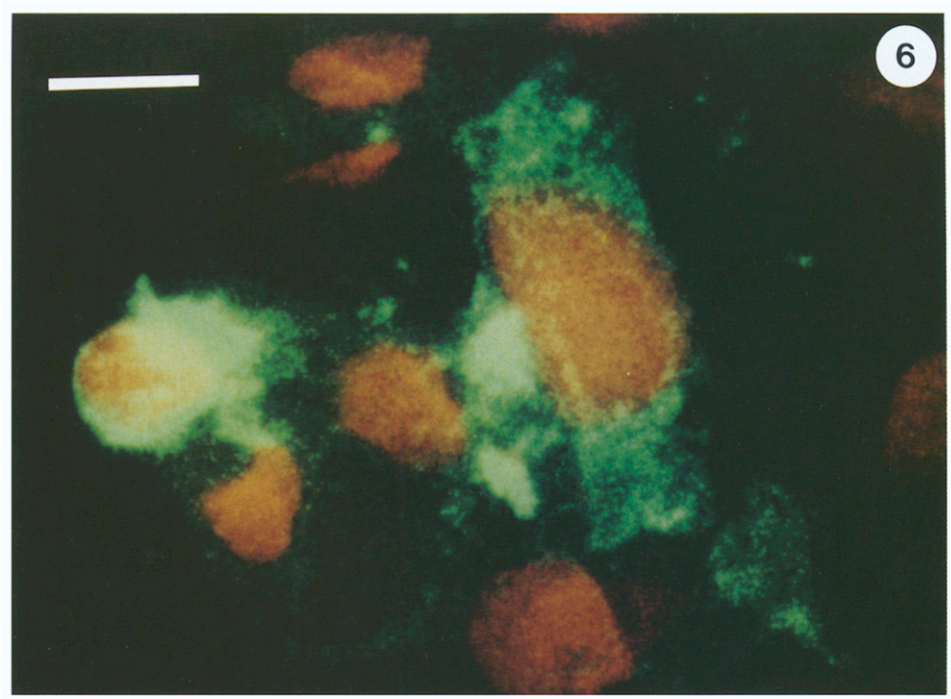


Fig. 4. Confocal micrograph showing effect of EDTA on corneal epithelium. The cornea was treated with EDTA at 1 mM concentration for 3 h. Note the loss of surface membrane and intensed nuclear labeling. Bar = 10 μm.

Fig. 5. Effect of digitonin on corneal epithelium. The cornea was treated with digitonin at 1 mM concentration for 3 h. Loss of surface plasma membrane is evident. Bar = 10 μm.



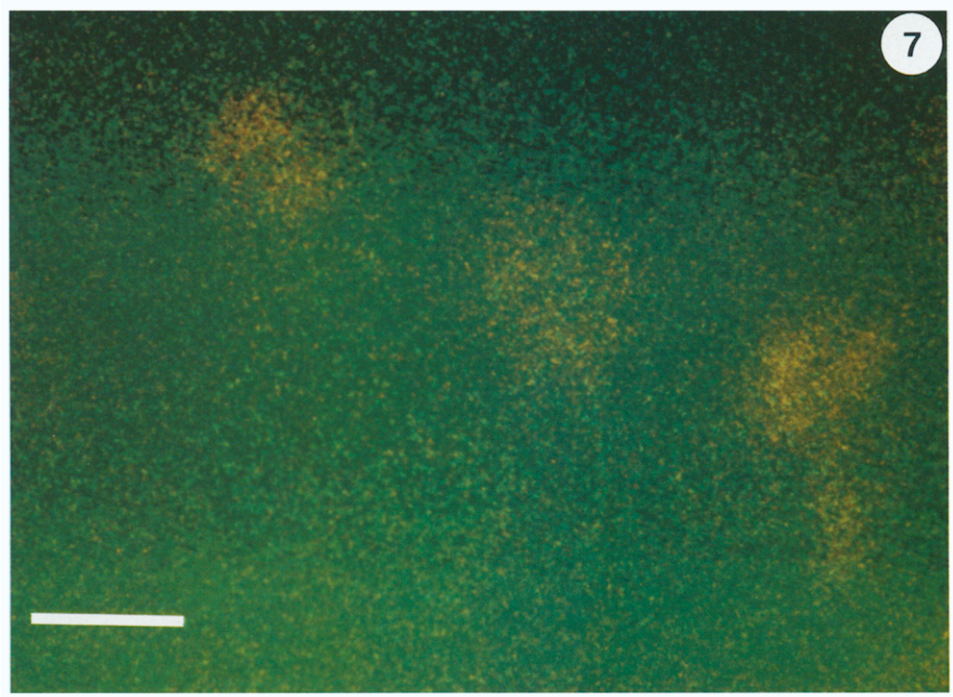


Fig. 6. Effect of sodium deoxycholate on corneal epithelium. The cornea was treated with sodium deoxycholate at 1 mM concentration for 3 h. Loss of surface membrane and intensed nuclear fluorescence, similar to that observed in EDTA and digitonin, are shown. Portions of plasma membrane (green label) remain to be seen. Bar = 10 μm.

Fig. 7. Effect of cytochalasin B on corneal epithelium. The cornea was treated with cytochalasin B at 1 mM concentration for 3 h. Nuclear labeling is minimal and plasma membrane is relatively preserved. Bar = 10 μm.

example, if no or minimal nuclear staining is observed, it may not necessarily mean that there is no damage but it may be because it takes time for the probe (PI, MW 668) to diffuse into the cells at a concentration high enough to allow detection. Thus, only the results at 3 h period are reported. For comparison purpose, a control cornea, similarly treated but with no enhancer, is used. Fig. 3 shows a confocal image of the superficial surface of the control cornea. As compared to most enhancer-treated corneas, the plasma membrane of the control is relatively preserved and the nuclear labeling is minimal. Severe membrane damage as indicated by the lack of membrane labeling is evident in the corneas treated with EDTA, digitonin, and deoxycholate (Figs. 4–6). The presence of nuclear labeling, and thus the nucleus, even without the presence of the cell membranes also suggests that structural components of the cells, i.e., certain types of the cytoskeletal filaments, are preserved which allows the nuclei to be retained in the cells. Surface cell separation which results in clustering of cells, clearly observed at low magnification, is also found in these tissues. This phenomenon is believed to result from the disrupting effect of the enhancers on the junctional components of the cells. In contrast to the above enhancers, cytochalasin B demonstrates negligible damage effect on the cell membranes. This effect is comparable to that observed in the control cornea. EDTA, a known calcium chelator, has been previously shown to promote absorption of a number of compounds in epithelia including the cornea (Kaye et al., 1968; Stern et al., 1981; Grass et al., 1985). Its mechanism is to interfere with the ability of calcium to maintain intercellular integrity (Simpson and Loenstein, 1977; Stern et al., 1981). The fact that EDTA disrupts plasma membrane structures is somewhat unexpected. Although the mechanism of this effect is not clear, there is evidence suggesting that EDTA, by chelating calcium, may cause structural breakdown of the cell cytoskeleton which in turn results in cell collapse and subsequent disintegration (Kaye et al., 1971; Kaye et al., 1974). Recently, Nishihata et al. (1985) have demonstrated that EDTA, like most surfactants, can cause leakage of cell proteins from rectal epithelia. Based on these findings, it appears that EDTA can exert multiple effects on the membrane. Like other surfactants, digitonin possesses certain detergent characteristics and the ability to permeabilize membranes in a wide variety of cells (Fiskum, 1985). In the cornea, digitonin has been found to selectively solubilize membrane cholesterol and cause exfoliation, layer by layer, of the epithelium (Wolosin, 1988). Bile salts, on the other hand, have been shown to promote peptide absorption not only by a direct solubilizing effect but also by inhibiting cellular enzymatic activity (Hirai et al., 1981). These authors also demonstrate that, at the same promoting efficacy, bile salts cause less solubilizing effect on the membrane. In the present study, despite the obvious membrane damage caused by the bile salt, the loss of plasma membrane appears to be less as compared to that observed in digitonin-treated cornea. This finding is in good agreement with results obtained from capacitance measurements, reported subsequently.

Cytochalasins are a group of small, naturally occurring heterocyclic compounds that bind specifically to actin microfilaments, the major component of the cell cytoskeleton, and alter their polymerization (Flanagan and Lin, 1980; Brown and Spudich, 1981). In addition to its normal role in regulating cell contractibility, mobility, and cell surface receptors, the cytoskeleton has been shown to participate in regulation of epithelial tight junction permeability (Craig and Pardo, 1979; Binder, 1980). Cereijido et al. (1980) demonstrated, using immunofluorescence microscopy, actin filament formation at the junctional complex of cultured epithelia. This microfilament was later found to play a role in positioning junctional strands, through its association with plasma membrane components, and influence the degree of opening of the occluding junctions (Meza, 1980). Among cytoskeleton-active agents, cytochalasin B is perhaps one of the most well-characterized agents and has been extensively used in studying the role of actin in various biological processes. Its specificity and efficacy on tight junction permeability makes it attractive for potential use in peptide delivery, especially for peptides that penetrate the tissue intercellularly. Results from this study show that cytochalasin B is effective in en-

hancing intercellular permeability in the cornea (see results on resistance measurements) with minimal membrane damage (Fig. 7).

Effect of penetration enhancers on membrane resistance and capacitance

To circumvent the potential problem of tissue polarization associated with direct current resistance measurements which has been found to cause an interfering effect on the determination of true membrane resistance (Lawler et al., 1960; Plutchik and Hirsch, 1963), the present work utilizes alternating current, by means of impedance measurements, to obtain membrane resistance. Impedance represents total electrical opposition to movement of charges which constitutes both resistive and capacitive components of the membrane. By measuring impedance, information on both membrane resistance and capacitance can be obtained. Since electrical resistance indicates charge flow across the membrane, it can be used as an index for ionic membrane permeability. On the other hand, capacitance reflects the ability of membrane dielectric to store charges, it can be used to indicate structural damage on the membrane surface. Previous work on the cornea (Rojanasakul and Robinson, 1990) has shown that changes in electrical resistance and capacitance correlate well with changes in the aqueous intercellular space and membrane surface integrity respectively.

In the present study, these two parameters are used to assess the effect of penetration enhancers on the membrane. Typical time-profiles for resistance and capacitance of the control cornea are shown in Fig. 8. Biphasic resistive behavior which has been previously shown to be responsible for changes in membrane potential and subsequent intercellular expansion (Rojanasakul and Robinson, 1990) is evident. This potential-dependent change occurs in the direction that promotes the preservation of normal membrane potential, i.e. the resistance increases if the cornea is depolarized and decreases if the cornea is hyperpolarized. The mechanism for this change is not yet clear, but presumably due to the change in tight junction permeability. A typical peak resistance value observed in this study is $\approx 800\ \Omega\ cm^2$. This value is

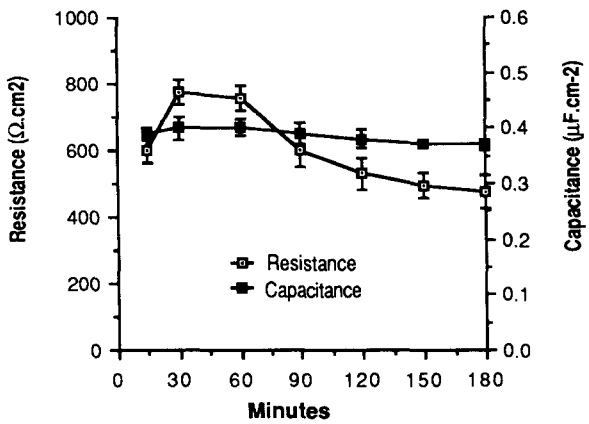


Fig. 8. Typical resistance and capacitance-time profiles of the cornea. A sine wave with current density of 10 $\mu A\ cm^{-2}$ was applied across the cornea and the resistance (1 Hz) and capacitance (500 Hz) were determined. Note the biphasic resistive and constant capacitive behavior of the cornea. Bars indicate 1 SE, $n = 6$.

relatively low as compared to those previously reported, i.e. $\approx 1000\ \Omega\ cm^2$ (Donn et al., 1959), ≈ 2 $k\Omega\ cm^2$ (Fischer and Zadunaisky, 1977), $\approx 9\ k\Omega$ cm^2 (Klyce and Wong, 1977), although a lower value of $\approx 600\ \Omega\ cm^2$ (Holt and Cogan, 1946) was also reported. This difference may be attributed to a number of factors; (a) maintenance of tissue integrity and viability, (b) animal variation, (c) experimental conditions, and (d) analytical techniques used. In the present study, tissue integrity and viability were carefully checked by means of electron microscopy and membrane potential measurements. Results of these studies (see Rojanasakul and Robinson, 1990) indicate the corneas remain viable with no detectable damage for up to 3 h. Previous results on fluorescent PI uptake also support these findings. Unlike the resistance, the corneal capacitance is remarkably constant, suggesting maintenance of membrane surface integrity during the perfusion study. The observed capacitance values (0.3–0.4 $\mu F\ cm^{-2}$) are slightly higher than those ($\approx 0.2\ \mu F\ cm^{-2}$), reported by Holt and Cogan (1946).

The effect of various penetration enhancers on the corneal resistance at concentrations ranging from 0.01 to 1.0 mM is shown in Figs 9–12. These results show that, for any enhancer, increasing the concentration results in a decrease in membrane

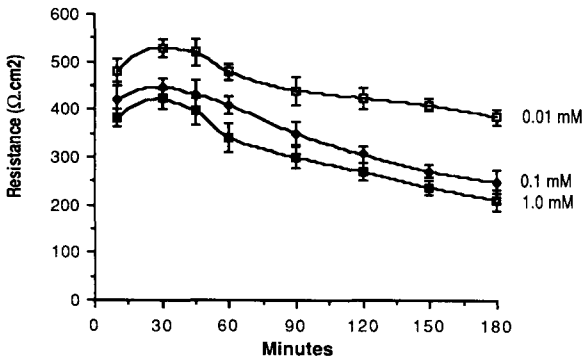


Fig. 9. Effect of dose of EDTA on corneal resistance. Positive correlation between concentration of EDTA and corneal resistive response is shown. Bars indicate 1 SE, $n = 4$.

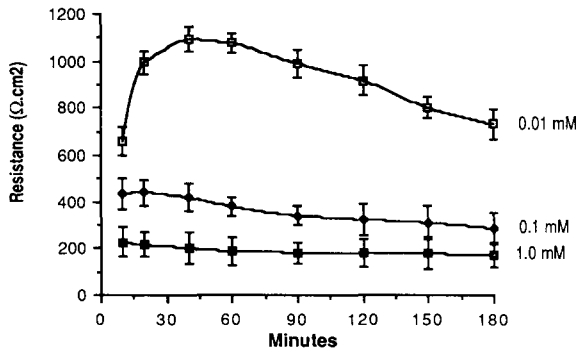


Fig. 10. Effect of dose of digitonin on corneal resistance. Positive resistive response occurs at high digitonin concentrations, i.e., 0.1 and 1.0 mM, while negative response occurs at low concentration, 0.01 mM (see control in Fig. 8). Bars indicate 1 SE, $n = 4$.

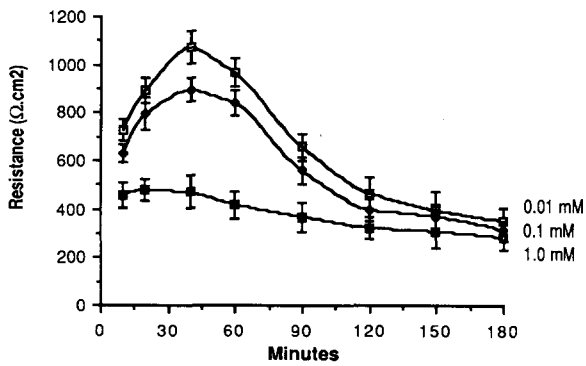


Fig. 11. Effect of dose of sodium deoxycholate on corneal resistance. Note the negative resistive response of the cornea during the first hour at deoxycholate concentrations of 0.01 and 0.1 mM. Bars indicate 1 SE, $n = 4$.

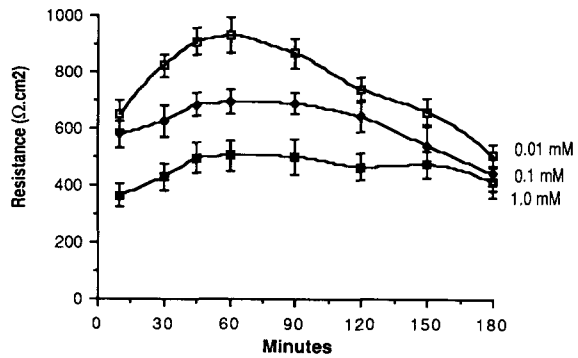


Fig. 12. Effect of cytochalasin B on corneal resistance. Note the negative resistive response of the cornea at enhancer concentration of 0.01 mM. Bars indicate 1 SE, $n = 4$.

resistance or, in other words, an increase in the membrane permeability. Interestingly, with the exception of EDTA, all enhancers exhibit both promoting and retarding effects on corneal permeability, depending on concentration. As compared to the control, digitonin (Fig. 10), sodium deoxycholate (Fig. 11), and cytochalasin B (Fig. 12) increase permeability at concentrations of $\approx$ 0.1–1 mM or higher while EDTA demonstrates such an effect at all concentrations used in this study. The negative effect of the enhancers at low concentration is not commonly known and their mechanisms are not yet known. However, this effect has been shown to occur in cytochalasin B-treated epithelia (Bentzel et al., 1980). These authors demonstrated, using freeze fracture electron

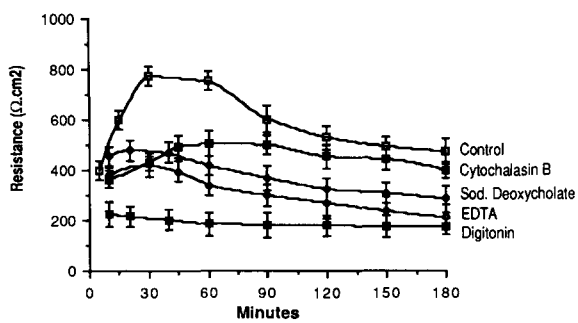


Fig. 13. Promoting efficacy of various penetration enhancers in the cornea. The effect of EDTA, digitonin, deoxycholate, and cytochalasin B, at fixed concentration (1 mM), on corneal resistivity is compared. Bars indicate 1 SE, $n = 4$.

microscopy, that this effect is facilitated through the cell cytoskeleton. At low concentrations, cytochalasin B disorganizes the orderly arranged junctional protein network and hampers molecular flow across the junction. At high concentrations, however, this agent disrupts junctional protein structures, and thus, facilitates flow through it.

A comparison between the relative efficacy of the promoting action of the enhancers is shown in Fig. 13. At an equimolar concentration of 1 mM, the rank order for efficacy is digitonin > EDTA > deoxycholate > cytochalasin B, with cytochalasin B being more effective than the bile salt and equally potent to EDTA in the first 30 min after administration. Results from capacitance measurements (Fig. 14), however, indicate gradual but a substantial drop in capacitance for digitonin, EDTA, and deoxycholate, suggesting membrane surface damage and its inability to store charges. Only a minimal drop in capacitance is observed for cytochalasin B. These results are in good agreement with the confocal microscopic observations. It, thus, appears that the increased permeability of the cornea presented by the first three enhancers may have resulted from a direct solubilizing effect of the plasma membrane, although EDTA may exert its effect on intercellular integrity as well. The effect of cytochalasin B is predominantly on the junctional portion of the epithelium. This agent and perhaps other cytoskeleton-active agents may be of potential use for safe and effective delivery of peptides, especially for those that permeate tissue intercellularly. Additional studies on the potential toxic effects of these compounds need also to be done if the compounds are to be used for practical purpose.

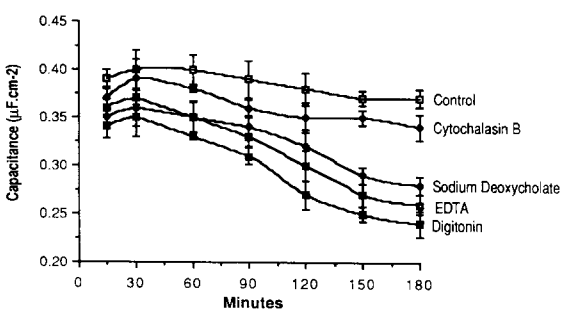


Fig. 14. Effect of penetration enhancers on corneal capacitance. Corneal capacitance as a function of time in the presence of EDTA, digitonin, deoxycholate, and cytochalasin B, at fixed concentration (1 mM) is shown. Bars indicate 1 SE, $n = 4$.

Conclusions

The present work investigates the promoting mechanisms of penetration enhancers in the cornea, utilizing direct confocal microscopic and quantitative electrical methods. In good agreement, the confocal and electrical results indicate a direct disrupting effect of the non-ionic surfactant, digitonin, bile salt, and EDTA on the corneal membrane. On the other hand, the effect of cytochalasin B is limited to the junctional portion of the epithelium. The cornea exhibits bi-directional resistive response to most enhancers with facilitative action at high concentration and inhibitive action at low concentration. With respect to enhancing power, digitonin is most effective, followed by EDTA, bile salt, and cytochalasin B. However, cytochalasin B appears to present the least harmful effect to the tissue. Its low toxicity along with high specificity on epithelial junction make this agent an attractive compound for enhanced peptide delivery.

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