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Effects of Chlorhexidine digluconate on Rotational Rate of *n*-(9-Anthroyloxy)stearic acid in Model Membranes of Total Lipids Extracted from *Porphyromonas gingivalis* Outer Membranes

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The purpose of this study was to provide a basis for studying the molecular mechanism of pharmacological action of chlorhexidine digluconate. Large unilamellar vesicles (OPGTL) were prepared with total lipids extracted from cultured *Porphyromonas gingivalis* outer membranes (OPG). The anthroyloxy probes were located at a graded series of depths inside a membrane, depending on its substitution position (*n*) in the aliphatic chain. Fluorescence polarization of *n*-(9-anthroyloxy)stearic acid was used to examine effects of chlorhexidine digluconate on differential rotational mobility, while changing the probes' substitution position (*n*) in the membrane phospholipids aliphatic chain. Magnitude of the rotational mobility of the intact six membrane components differed depending on the substitution position in the descending order of 16-(9-anthroyloxy)palmitic acid (16-AP), 12, 9, 6, 3 and 2-(9-anthroyloxy)stearic acid (12-AS, 9-AS, 6-AS, 3-AS and 2-AS). Chlorhexidine digluconate increased in a dose-dependent manner the rate of rotational mobility of hydrocarbon interior of the OPGTL prepared with total lipids extracted from cultured OPG, but decreased the mobility of membrane interface of the OPGTL. Disordering or ordering effects of chlorhexidine digluconate on membrane lipids may be responsible for some, but not all of its bacteriostatic and bactericidal actions.

Key Words: Liposomes, Chlorhexidine digluconate, Fluorescence probe technique, Membrane hydrocarbon interior, Membrane interface, Rotational rate, *n*-(9-anthroyloxy)stearic acid

INTRODUCTION

It is known that chlorhexidine is a potent membrane-active agent against both Gram-positive and Gram-negative bacteria (Hugo, 1978), causes membrane damage to neutrophils (Kenney et al, 1972; Knuutilla & Sodering, 1981), produces deleterious membrane perturbations in blood cells (Gabler et al, 1987) and has a decreasing effect on the epithelial-cell-lipid packing order (Audus et al, 1992). Also, Tsuchiya (1999) reported that the antiplaque effect of chlorhexidine is due to the reduction in membrane fluidity of both hydrophilic and hydrophobic regions. However, the precise location of pharmacological action of chlorhexidine has been a continuously controversial subject.

The difficulty with the membrane fluidity concept is that any physical parameter chosen will be a property of the spectroscopic method employed, specifically its particular time window (from $\sim 10^{-5}$ sec for NMR to $\sim 10^{-9}$ sec for fluorescence and ESR) and the properties of the probe (shape, charge, location etc) (Stubbs & Rubin, 1993). It is highly likely that bulk or average spectroscopic properties of cell membranes may not be useful in building a hypothesis for the mechanism of pharmacological action of chlorhexidine on bacteriostatic or bactericidal effects. Earlier studies (Kenney et al, 1972; Hugo, 1978; Knuutilla & Södering, 1981; Gabler et al, 1987; Audus et al, 1992) on effects of chlorhexidine on membrane bulk lipids have examined the average or total change in the lipid environment. However, with few exceptions (Tsuchiya, 1999; Jang et al, 2003), little attention has been given to the effect of chlorhexidine on the fluidity of special domain of anaerobic bacterial (in particular, gingivitis causative microorganisms) and model membranes.

Earlier studies have shown that the fluorophores of anthroyloxy derivatives located at a graded series of levels from the surface to the center of the lipid bilayer structure (or a series of anthroyloxy fatty acids indicates that the depth of the group is almost linearly related to the number of carbon atoms between it and the carboxyl group) (Tilley et al, 1979; Villalaín & Prieto, 1991; Abrams et al, 1992;

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ABBREVIATIONS: PG, *Porphyromonas gingivalis*; OPGTL, total lipids extracted from cultured *Porphyromonas gingivalis* outer membranes; *n*-AS, *n*-(9-anthroyloxy)stearic acid.

Abrams & London, 1993; Mason, 1994). The anthroyloxy stearate (AS) probes can also be used to differentiate whether the bilayer has a fluidity gradient across it, as the anthroyloxy group can be positioned at different positions of the stearic acid moiety (Thulborn et al, 1979; Molitoris & Hoilien, 1987; Villalaín & Prieto, 1991; Abrams & London, 1993). These probes have been suggested to measure primarily the dynamic component of membrane fluidity (Vincent et al, 1982; Schachter, 1984; Molitoris & Hoilien, 1987).

We have reported that chlorhexidine digluconate increased the rate of rotational mobility of hydrocarbon interior of the cultured *Porphyromonas gingivalis* outer membranes (OPG) in a dose-dependent manner, however, decreased the mobility of membrane interface of the OPG. If chlorhexidine digluconate acts on a specific monolayer of model membranes with no proteins, then, where does it exert its predominant effects between the hydrophilic and hydrophobic regions and where does it exert its predominant effects among phospholipid aliphatic chains of carbon atom numbers of 2, 3, 6, 9, 12 and 16? It is the aim of this study to provide a basis for studying the molecular mechanism of pharmacological action of chlorhexidine, by investigating the effect of chlorhexidine digluconate on differential rotational rate among the number of carbon atoms in the model membranes phospholipids (OPGTL), prepared with total lipids extracted from *Porphyromonas gingivalis* (PG) outer membrane phospholipids which differ in rotational mobility. Employing fluorescence polarization of 16-(9-anthroyloxy)palmitic acid (16-AP), 12-(9-anthroyloxy) stearic acid (12-AS), 9-(9-anthroyloxy)stearic acid (9-AS), 6-(9-anthroyloxy)stearic acid (6-AS), 3-(9-anthroyloxy)stearic acid (3-AS) and 2-(9-anthroyloxy)stearic acid (2-AS), we investigated the effect of chlorhexidine digluconate on rotational mobility of the OPGTL outer monolayers.

METHODS

Materials

The fluorescent probes, 16-AP, 12-AS, 9-AS, 6-AS, 3-AS and 2-AS, were purchased from Molecular Probes, Inc. (Junction City, OR, USA), and chlorhexidine digluconate and *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (Hepes) were from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were purchased commercially and were of the highest quality available. Water was deionized.

Bacterial growth conditions

ATCC 33277 (PG) was obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured as described previously (Lamont et al, 1995). PG was maintained as frozen stock cultures and grown anaerobically (Bactron IV Anaerobic Chamber, Sheldon Manufacturing Inc., OR, USA) in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with 1% (wt/vol) yeast extract, 0.0005% (wt/vol) hemin, and 0.0001% (wt/vol) menadione at 37°C for 3 days in an atmosphere of 80% N_2, 10% CO_2, and 10% H_2.

Preparation of outer membranes

The procedure was essentially as described by Smalley et al. (1993). Briefly, freeze-dried whole cells (200 mg) were suspended in 60 ml of 0.14 M NaCl containing 10 mg of EDTA (pH 7.3), and the suspension was incubated for 30 min at 37°C with stirring (Smalley & Birss, 1987). This serves to dissociate OPG and to inhibit any possible cell associated protease activity (Tsutsui et al, 1987) during preparation. After passing through a 25 gauge needle twice, the cells were pelleted by centrifugation (20,000$\times$g for 30 min, 4°C), and the supernatant was taken as crude OPG preparation. Residual EDTA and buffer salts were removed by dialysis against distilled water for 4 h at 4°C, and OPG fraction was then freeze-dried. The protein concentration was determined by the method of Lowry et al. (1951), using bovine serum albumin (BSA) as a standard.

Extraction of total lipids from OPG

Total lipids were extracted by the methods of Folch et al. (1956), Madeira & Antunes-Madeira (1976) and Yun & Kang (1990). The OPG were homogenized with 2 : 1 chloroform-methanol mixture (v/v), followed by filtering homogenate. In order to purify the crude extracts, the filtrate was mixed throughly with 0.2 its volume of either water or an adequate salt solution. The lower phase was saved and evaporated to dryness under nitrogen at 37°C, and the residue was taken up in a small volume of chloroform.

Preparation of model membranes

Large unilamellar liposomes (0.7 mg of total lipids/ml, pH 7.4) were prepared by the method previously described (Yun & Kang, 1992). The total lipids in chloroform solution were deposited on the sides of a round-bottom flask by removing the organic solvent through rotary evaporation. The total lipids were then redissolved in diethyl ether which had been redistilled in the presence of $NaHSO_3$ immediately prior to use. Phosphate buffered saline (PBS: 8 g/l NaCl, 0.2 g/l KCl, 0.2 g/l KH_2PO_4, 1.15 g/l $Na_2HPO_4 \cdot 7H_2O$, 0.48 g/l Hepes, pH 7.4) was added to the solution of total lipids, and the organic/aqueous mixture was placed in an ultrasonic processor (Sonics & Materials, Inc., Danbury, CT.) under N_2 at 30°C. It was sonicated for 5 min to form a milky white homogeneous emulsion. The emulsion was then transferred to a rotary evaporator, and the organic solvent was removed under reduced pressure. During evaporation of the solvent, the system foamed, and as the process continued, progressively higher vacuum was applied to maintain foaming. As the majority of the solvent were removed, the material first formed a viscous gel, and subsequently (within 5~10 min) it became an aqueous suspension. At this time, additional PBS was added, and the preparation foamed and was vented again several times until the foaming ceased. The procedure was finished when no foaming occurred. The preparation was then dialyzed and passed through a Sepharose 4B column.

Fluorescence measurements

Fluorescence measurements were taken using a modified method of earlier studies (Mollitoris et al, 1985; Mollitoris & Hoilien, 1987). The OPGTL was suspended in PBS to 0.7 mg lipid/ml concentration. Stock solutions of the probes in methanol (2×10^{-5} M) were prepared and kept in a cold dark place. Aliquots were added to the suspensions of the

Table 1. Fluorescence parameter of 16-(9-anthroyloxy)palmitic acid (16-AP), 12-(9-anthroyloxy)stearic acid (12-AS), 9-(9-anthroyloxy)stearic acid (9-AS), 6-(9-anthroyloxy)stearic acid (6-AS), 3-(9-anthroyloxy)stearic acid (3-AS) and 2-(9-anthroyloxy)stearic acid (2-AS) in model membranes (OPGTL) of total lipids extracted from cultured *Porphyromonas gingivalis* outer membranes (OPG)

Membrane	Parameter	16-AP	12-AS	9-AS	6-AS	3-AS	2-AS
OPGTL	anisotropy	0.086±0.001	0.093±0.002	0.104±0.002	0.111±0.001	0.119±0.001	0.124±0.002

Fluorescence measurements were performed at 37°C (pH 7.4). Values are represented as mean±SEM of 5 determinations.

OPGTL, so that the final concentrations of the probes were 4×10^{-8} M probes incorporated. The mixture was stirred for 20 min at room temperature in order to reduce the concentration of methanol that might alter the rotational rate of the outer monolayer of the OPGTL. Also, the mixtures were bubbled with dry nitrogen for 1 min with 20 min intervals in order to eliminate oxygen that might act as a quencher. Concentrated solutions of chlorhexidine digluconate were prepared in PBS and added to the labeled model membrane suspension to give the desired concentration of the drug. The pH of the buffered sample was not changed significantly by addition of the drug.

Fluorescence measurements were carried out with a Multi Frequency Cross-Correlation Phase and Modulation Fluorometer (ISS K2-003), equipped with a thermostated cell holder and performed at pH 7.4 (37±0.1°C). The fluorescent probes were excited at 360 nm (4 nm slit width) and emissions were recorded at 445 nm (8 nm slit width) through a sharp cut-off filter (Schott KV418). Corrections for light scattering (membrane suspensions without fluorescent probes) and for fluorescence in the ambient medium (quantified by pelleting the membranes after each estimation) were routinely made, and the combined corrections were less than 7% of the total fluorescence intensity observed for anthroyloxystearate-loaded suspensions. The intensity of the components of the fluorescence which was parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the direction of the vertically polarized excitation light was determined by measuring the emitted light through polarizers oriented vertically and horizontally. Polarization (P) was obtained from intensity measurements using $P=(I_{\parallel}-GI_{\perp})/(I_{\parallel}+GI_{\perp})$, where G is a grating correction factor for the monochromator's transmission efficiency for vertically and horizontally polarized light. This value is given by the ratio of the fluorescence intensities of the vertical to horizontal components, when the exciting light is polarized in the horizontal direction. The polarization was expressed as anisotropy [$r=2P/(3-P)$] of the probes.

Data analysis

Each value was expressed as mean±SEM (n=5 for all experiments). Differences in anisotropy (r) were statistically analyzed by the Student's t-test.

RESULTS

In the present study, using the fluorescence probe technique, we evaluated differential effects of dicationic chlorhexidine digluconate on the differential rotational mobility among the number of carbon atoms in the OPGTL phospholipids which differ in rotational mobility. In order to determine the effect of the chlorhexidine digluconate on the rotational mobility rate, it was first necessary to demonstrate that the drug did not interact directly with fluorescent probes, thereby quenching its fluorescence. Quenching of absorbance-corrected fluorescence intensity of 16-AP, 12-AS, 9-AS, 6-AS, 3-AS and 2-AS in OPGTL by the chlorhexidine digluconate was not observed at all of the concentration levels at which the drug was tested. Hence, the possibility of direct quenching of fluorescence of the probes by the drug was ruled out.

As shown in Table 1, the anisotropy (r) values of 16-AP, 12-AS, 9-AS, 6-AS, 3-AS and 2-AS in the outer monolayer of intact OPGTL at 37°C (pH 7.4) indicate that the rate of rotational mobility of hydrocarbon interior is faster than that of the model membrane interface. The rotational mobility's degrees of the number of phospholipid carbon atoms differed, depending on the carbon numbers, in the descending order of the 16-AP, 12-AS, 9-AS, 6-AS, 3-AS and 2-AS.

The chlorhexidine digluconate may induce disordering or ordering in their host lipids. Ordering takes place in the membrane interface, whereas disordering occurs deep within the acyl chains. If the degree of disordering or ordering effects on the membranes' outer monolayer by chlorhexidine digluconate is expressed in percentage, the magnitude of ordering effects induced by chlorhexidine digluconate on the membrane surface region (in the case of 2-AS) is smaller than that of disordering effects (in the cases of 16-AP and 12-AS) induced by the drug on the membrane hydrocarbon interior. However, when the altered anisotropy (r) values were converted to temperature, the magnitude of ordering effects induced by chlorhexidine digluconate on the membrane interface was found to be greater than that of disordering effects induced by the drug on membrane hydrocarbon interior [As to the method to convert the anisotropy (r) values to temperature, refer to the following section].

In the present study, the sensitivities to the increasing or decreasing effects on the rotational mobility of hydrocarbon interior or membrane interface of OPGTL were higher than those of OPG (Jang et al, 2003).

Disordering effects of chlorhexidine digluconate on rotational rate in the monolayer hydrocarbon interior

We recently reported that the disordering effects of chlorhexidine digluconate on the hydrocarbon interiors of outer monolayers of bacterial outer membranes increases roughly proportionally with its depth of site of action (the more effective penetration into hydrocarbon interior could result in higher disordering effects on the model membrane outer monolayer) (Jang et al, 2003). The effects of increasing concentrations of chlorhexidine digluconate on the anisotropy (r) of the probes in hydrocarbon interior of the monolayer of OPGTL are shown in Fig. 1. The chlorhexidine

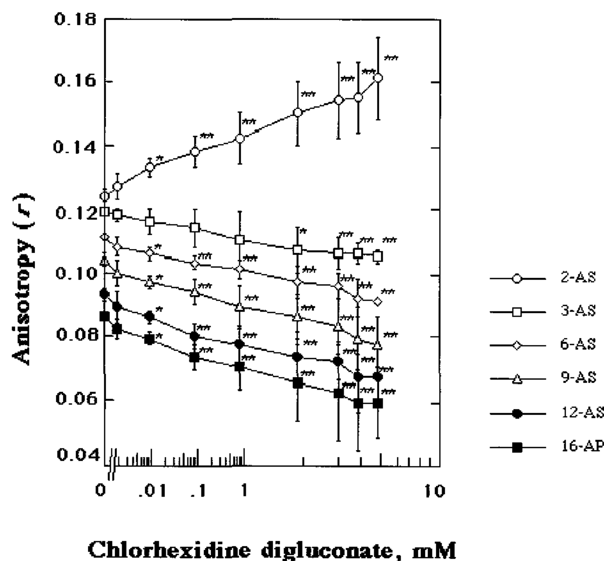

Fig. 1. Effects of chlorhexidine digluconate on the anisotropy (r) of 16-(9-anthroyloxy)palmitic acid (16-AP), 12-(9-anthroyloxy)stearic acid (12-AS), 9-(9-anthroyloxy)stearic acid (9-AS), 6-(9-anthroyloxy)stearic acid (6-AS), 3-(9-anthroyloxy)stearic acid (3-AS) and 2-(9-anthroyloxy)stearic acid (2-AS) in model membranes (OPGTL) of total lipids extracted from cultured *Porphyromonas gingivalis* outer membranes (OPG). Fluorescence measurements were performed at 37°C (pH 7.4). Each point represents mean±SEM of 5 determinations. An asterisk and double asterisk signify $P<0.05$ and $P<0.01$, respectively, compared to control according to Student's t-test.

digluconate decreased the anisotropy (r) of the probes (increased rate of rotational mobility) in the monolayer of the OPGTL in a concentration-dependent manner. The significant decreases in the anisotropy (r) values of 16-AP, 12-AS and 9-AS by the drug were observed at all used concentration levels (0.01, 0.10, 1.00, 2.00, 3.00, 4.00 and 5.00 mM), except 0.001 mM level (Fig. 1).

The difference in the anisotropy (r) value of 12-AS found in hydrocarbon interior of the monolayer of OPGTL before and after adding 0.10 mM chlorhexidine digluconate was 0.013. This can be illustrated by comparing the effects of temperature on this parameter. The anisotropy (r) of 12-AS in hydrocarbon interior of the monolayer of OPGTL was 0.093±0.002 at 37°C (pH 7.4) and 0.125±0.002 at 25°C (pH 7.4). Thus, the difference of 0.013 in the anisotropy (r) values of 12-AS was similar to that produced by raising approximate 4.9°C temperature. In the present study, increasing effect of rotational mobility of chlorhexidine digluconate on the hydrocarbon interior of OPGTL outer monolayer was far greater than the drug's effect on the OPG outer monolayer (Jang et al, 2003).

Ordering effects of chlorhexidine digluconate on rotational rate of the monolayer surface region

We recently reported that chlorhexidine digluconate increased the anisotropy (r) of 2-AS in the OPG membrane interface (Jang et al, 2003). The effects of increasing concentrations of chlorhexidine digluconate on the anisotropy (r) of 2-AS in membrane interface of the monolayer of OPGTL are shown in Fig. 1. Chlorhexidine digluconate increased the anisotropy (r) of 2-AS (decreased rate of rotational mobility) in a concentration-dependent manner. The significant increases in the anisotropy (r) values by the drug were observed at all the concentration levels used (0.01, 0.10, 1.00, 2.00, 3.00, 4.00 and 5.00 mM), except 0.001 mM level (Fig. 1).

The difference in the anisotropy (r) values of 2-AS found in membrane interface of the monolayer of OPGTL before and after adding 0.10 mM chlorhexidine digluconate was 0.014. This can be illustrated by comparing the effects of temperature on this parameter. The anisotropy (r) of 2-AS in membrane interface of the monolayer of OPGTL was 0.124±0.002 at 37°C (pH 7.4), and 0.151±0.003 at 25°C (pH 7.4). Thus, the difference of 0.014 in the anisotropy (r) value of 2-AS in the membrane interface was the same as that produced by the fall of approximate 6.2°C temperature. Decreasing effect of rotational mobility of chlorhexidine digluconate on the membrane interface of the OPGTL was also far greater than the drug's effect on the OPG membrane interface (Jang et al, 2003).

DISCUSSION

The precise location of pharmacological action of chlorhexidine at molecular level has been a continuously controversial subject. The current consensus is that chlorhexidine has a site(s) of action located within the cell membrane, presumably on the inner and outer membranes.

In regard to 2-AS distribution region, the study by Villalaín & Prieto (1991) is of special interest among several studies (Thulborn et al, 1979; Vincent et al, 1982; Schachter, 1984; Molitoris & Hoilien, 1987). They reported that 2-AS is a peculiar member of the family of probes, since the chromophore is adsorbed on the membrane interface (Villalaín & Prieto, 1991). Membrane interface is difficult to precisely define, and it certainly includes the polar region near the phospholipid head groups or even the carbonyl groups which are largely involved in hydrogen bonds. 16-AP, 12-AS, 9-AS, 6-AS and 3-AS are distributed in the hydrophobic interior of the cell membrane outer monolayer (Thulborn et al, 1979; Vincent et al, 1982; Schachter, 1984; Molitoris & Hoilien, 1987). The AS probes can be used to differentiate whether the bilayer has a fluidity gradient across it, because the anthroyloxy group can be positioned at different positions of the stearic acid moiety (Thulborn et al, 1979; Vincent et al, 1982; Schachter, 1984; Molitoris & Hoilien, 1987; Villalaín & Prieto, 1991). These probes have been suggested to primarily prove the dynamic component of membrane fluidity (Vincent et al, 1982; Schachter, 1984; Molitoris & Hoilien, 1987). Since rotational mobility rate of membrane lipid bilayers can be measured by a rather simple method without measuring life time of a fluorescent probe, it is economical. To the best of our knowledge, this is the first study to apply the fluorescence probe technique to liposomes and to measure the rotational rate of the aliphatic chains of the liposome outer monolayer.

Our data presented herein showed that, even at physiologically relevant concentrations (Buck et al, 2001; Gerlach & White, 2001; Spratt et al, 2001), chlorhexidine digluconate increased or decreased the rate of hydrocarbon interior or membrane interface of the monolayer of OPGTL. Using the membrane interface probe 2-AS, we found that chlorhexidine digluconate decreased the rotational mobility of lipids in the interface of OPGTL, whereas chlorhexidine

digluconate increased the rotational mobility of the probes (16-AP, 12-AS, 9-AS, 6-AS and 3-AS) in the hydrocarbon interior of the model membrane. This is due to differences in the intrinsic component and/or the structure in surface and hydrocarbon region of the monolayer of model membranes. The precise mechanism of the action(s) of the chlorhexidine digluconate as to its ordering and disordering effects on the monolayer of OPGTL is not yet known, however, the following possible mechanisms might be considered.

The chlorhexidine digluconate binds (the competitive binding of the chlorhexidine digluconate and water) strongly to the phosphate moiety of phospholipids in the outer monolayer surface and weakly to the carbonyl group in competition with water in the monolayer, and effectively establish formation of hydrogen bonds with the carbonyl moiety, which is associated with significant change in hydration of the chlorhexidine digluconate molecule itself. The incorporation of the chlorhexidine digluconate into the model membranes causes alterations of the surface charge density of the membrane's outer monolayer and then a conformational change in the phospholipid head groups. At the same time, they may exert a significant influence on hydration of the lipid bilayer. Fisher et al (1975) and Fisher & Quintana (1975) have demonstrated that ion-ion interactions take place between the dicationic chlorhexidine molecules and anionic carboxylate groups in stearic acid monolayers at pH of 5.0~6.0 (but not at pH 3), and that the hexamethylene hydrophobic chain of the biguanide is constrained at the cell surface. They further demonstrated the ability of chlorhexidine to anchor to the polar head groups of the film-forming molecules. These results may explain the hydrophobicity- increasing effect of chlorhexidine on the bacterial cell surface (Fisher et al, 1975; Fisher & Quintana, 1975). Consequently, such competitive binding decreases the rate of rotational mobility and increases hydrophobicity. The interaction of the dicationic chlorhexidine digluconate with the outer monolayer's hydrocarbon region may rearrange the intermolecular hydrogen-bonded network among phospholipid molecules, liberating hydrated water molecules on the monolayer of the model membranes. The interaction may also change the orientation of the P-N dipole of phospholipid molecules. These changes should cause disordering of the hydrocarbon interior of the monolayer. Thus, disordering and ordering effects of the chlorhexidine digluconate could affect the transport of small molecules in bacterial outer membranes, leading to the bacteriostatic or bactericidal action.

Tsuchiya (1999) reported that chlorhexidine increased fluorescence polarization of both 1-anilinonaphthalene-8-sulfonic acid (ANS) and *N*-phenyl-1-naphthylamine (PNA) in both liposomes of dipalmitoylphosphatidylcholine (DPPC) and dioleoylphosphatidylcholine (DOPC). Although the polarization of PNA in the DPPC liposomes was exceptionally decreased, chlorhexidine at $1\sim100\,\mu$M/l increased the polarization of ANS and PNA in the 50 mol% DPPC and 50 mol% DOPC liposomes. The results of Tsuchiya (1999) are not consistent with our present results. However, Tsuchiya's results are concordant with the results of the study about the effects of chlorhexidine on fluidity of the DPPC hydrocarbon interior, employing the florescent probe PNA and the results of the study about the chlorhexidine effects on fluidity of both DPPC and DOPC surface region, employing the fluorescent probe ANS. These differences cannot be fully explained.

The questions of whether chlorhexidine digluconate interferes with membrane protein function by directly binding to the proteins or whether the main modes of action occurs indirectly through a change in the physicochemical properties of the lipid membranes into which the chlorhexidine digluconate readily diffused remain controversial. Because biological membranes are of highly complex composition, it has not been possible to monitor changes in the local lipid environment and to determine its effect on the membrane protein function at the same time.

It is possible to explain the multiplication effects by citing the increased or decreased mobility of protein triggered by lipids, however the reverse situation of protein triggering change in lipids is more likely (Jang et al, 2003). It is certain that chlorhexidine digluconate increases or decreases the mobility of the outer monolayer of bacterial outer membrane, but the direct effects of chlorhexidine digluconate on protein appear to have such effects magnified on the lipid (Jang et al, 2003). That is to say, before, during or even after the interaction of the chlorhexidine digluconate with proteins, the fluidization of membrane lipids may provide an ideal microenvironment for optimum bateriostatic or bactericidal effects. In conclusion, the present data together with the results of Jang et al. (2003) suggest that chlorhexidine digluconate interacts (directly) with membrane lipids, affects the fluidity of the membrane, and exerts some influence on the proteins which associate tightly with membrane lipids through covalent and noncovalent bonds.

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