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Ammonium glycyrrhizinate (AMGZ) effects on membrane integrity

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Summary

Potential mechanisms by which ammonium glycyrrhizinate (AMGZ) modifies biomembranes and promotes transcellular permeation of low molecular weight markers were investigated. The effects of increasing AMGZ concentration, pH, and ionic strength on erythrocyte hemolysis and on the steady-state fluorescence anisotropy, r, of fluorophore-labeled tracheal and erythrocyte ghost membranes were studied. At AMGZ concentrations greater than 1%, significant hemolytic activity was observed. Hemolysis was not observed over either the pH or the ionic strength ranges produced by corresponding concentrations of AMGZ. A significant decrease in r, a measure of lipid packing order, was observed in erythrocyte ghost membranes at AMGZ concentrations greater than 0.5 and 1.0% AMGZ in both lipid-water interfaces labeled with 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH) and core lipid domains labeled with 1,6-diphenyl-1,3,5-hexatriene (DPH), respectively. Fluorescence lifetime studies indicated that the DPH and TMA-DPH probes were to some degree sensitive to AMGZ and suggested the possibility of some contributions of direct AMGZ : probe interaction to interpretation of the fluorescence studies. A significant decrease in lipid packing order was also observed predominantly at the lipid-water interace of apical tracheal cell membranes at AMGZ concentrations greater than 0.5%. The lipid packing order for bovine trachea apical membranes and erythrocyte ghosts was not affected by varied pH or ionic strength produced by corresponding AMGZ additions. Collectively, results suggested that direct effects of AMGZ, as opposed to accompanying pH or ionic strength changes, on primarily membrane components at the lipid-water interface may contribute to alterations in membrane permeability to low molecular weight substances.

Introduction

Several classes of absorption promoters have been found to alter the structural integrity of membranes, one of several mechanisms that can promote enhanced cell and tissue permeability to various substances (Sarkar, 1992). Therefore, the permeability enhancement of many absorption promoters has been related to the ability to either lyse erythrocytes (Hirai et al., 1981; Mishima et al., 1989) or to the molecular packing of the lipids in biomembranes (Kajii et al., 1985, 1986, 1988; Iseki et al., 1988; LeCluyse et al., 1991). Although the latter effects are more often subtle, and in some instances are associated with no gross

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changes in tissue morphology, the promoter-induced changes in membrane lipid packing order can be correlated with promoter-enhanced absorption potency of some agents (Kajii et al., 1985, 1986, 1988; Iseki et al., 1988; LeCluyse et al., 1991). Alterations in lipid packing order can contribute to membrane permeability alterations through direct disruptions of membrane integrity (i.e., decreased lipid packing order often referred to as increased 'membrane fluidity') and altering lipid:protein interactions necessary for the normal function of membrane enzymes and transport or carrier mechanisms (Audus, 1992; Sarkar, 1992).

Previous investigations demonstrated that ammonium glycyrrhizinate (AMGZ), the ammonium salt of glycyrrhetinic acid glucoside, increased the permeation of low molecular weight markers, mannitol and Lucifer yellow, across ovine nasal mucosa in vitro without gross morphological damage to the tissue (Reardon et al., 1993). Currently, the permeability promoting mechanism of AMGZ on airway epithelium is unknown. The objective of this study was to determine the effects of AMGZ on the perturbation of biomembranes. This objective was achieved by (1) studying the effects of varying AMGZ concentration, pH, and ionic strength on erythrocyte hemolysis, and (2) studying the effects of varying AMGZ concentration, pH, and ionic strength on the lipid packing order of erythrocyte ghosts and airway epithelial membranes employing steady-state fluorescence spectroscopic techniques. The pHs and ionic strengths investigated spanned the range for solutions of AMGZ at concentrations enhancing absorption in nasal epithelia. As a representative cell type of the epithelia of nasal, tracheal and upper airway mucosa, apical bovine tracheal epithelial cell membranes were isolated and examined in this study.

Materials and Methods

Materials

1,6-Diphenyl-1,3,5-hexatriene (DPH) and 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH) were purchased from Molecular Probes (Eugene, OR). Bio-Rad Protein Assay was purchased from Bio-Rad (Richmond, CS). Ammonium glycyrrhizinate (AMGZ) was purchased from Sigma Chemical Co., St. Louis, MO. All other reagents were of the highest grade commercially available.

Human erythrocyte isolation

Erythrocyte isolations were performed by a modification of a previously described procedure (Hirai et al., 1981). Briefly, venous human blood from healthy, nonsmoking volunteers was collected in tubes with EDTA as the anticoagulant for a total volume of 50 ml. The blood was then centrifuged for 20 min at $1000 \times g$ at 4°C. All subsequent steps were performed at 4°C. The plasma and buffy coat were removed by aspiration. The red blood cells were resuspended with an isotonic Ringer's solution comprised of 112 mM NaCl, 5.0 mM KCl, 1.2 mM $CaCl_2$, 2.6 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 1.6 mM Na_2HPO_4, 25 mM $NaHCO_3$, 10 mM Hepes, 1 mM dithiothreitol (DTT), 95% O_2/5% CO_2, pH 7.4 (modified BBRS) to achieve the original volume (50 ml) and washed three times for 20 min at $1000 \times g$. The washed erythrocytes were then diluted with modified BBRS to produce 50 ml of the erythrocyte suspension.

Hemolysis study

Erythrocyte hemolysis experiments were performed by a modification of a previously described procedure (Mishima et al., 1989). 5 ml of the erythrocyte suspension, prepared as above, was diluted with modifed BBRS to achieve a final volume of 50 ml. A hypotonic solution of 50 mM NaCl was prepared as the hemolytic control (Robak and Duniec, 1986). Test solutions were prepared by dissolving AMGZ in modified BBRS to achieve 0.25–2.5% AMGZ concentrations (w/v). Ionic strength solutions were prepared by adding concentrated NaCl to modified BBRS to achieve the indicated ionic strengths. The pH solutions were prepared by adjusting the pH of modified BBRS with HCl and NaOH.

2 ml of the indicated hypotonic, AMGZ, pH or ionic strength solution was added to clean

borosilicate culture tubes. Then, 0.2 ml of the diluted erythrocyte suspension was added to each tube. The tubes were incubated for 30 min at 37°C. Following the incubation, the mixture was centrifuged in a microcentrifuge for 5 min, and the absorbance of the supernatant was measured at 543 nm. The hypotonic solution of 50 mM NaCl was used as the control to calculate the percent control for AMGZ, pH, and ionic strength solutions.

Human erythrocyte ghost preparation

Erythrocyte ghost preparation was performed by a modification of a previously described procedure (Dodge et al., 1963). Briefly, hemolysis of the human erythrocytes was performed by adding ultrapure distilled water (hypotonic) to the erythrocyte suspension, prepared above, in an approximate 10:1 ratio. The contents were then gently mixed and centrifuged at 20000 $\times g$ for 40 min at 4°C. The supernatant was carefully decanted, and the pellet resuspended in ultrapure water to obtain the original volume, and centrifuged as before three to four times until the supernatant was clear or slightly pink. Since the pellet was loose, the supernatant was carefully poured off, and the erythrocyte button was added to 250 ml of 0.1 N KOH. The erythrocyte suspension was allowed to sit overnight at room temperature to form a homogeneous erythrocyte ghost suspension. The erythrocyte ghost suspension was washed with modified BBRS three times at 1000 $\times g$ at 4°C and resuspended with modified BBRS to achieve a final volume of 10 ml.

Bovine trachea apical membrane preparation

Bovine trachea apical membrane preparation was performed by a modification of the previously described procedures (Langridge-Smith et al., 1983; Fong et al., 1988). Bovine trachea obtained from a local slaughterhouse was removed 10–15 min after each animal had been killed and immediately put on ice for transportation. Each trachea was divided into two or three segments, opened anteriorly, and washed in ice-cold BBRS containing 1 mM DTT to remove debris and mucus from the lumen. The strips of mucosa with underlying connective tissue and cartilage were incubated for 15 min in ice-cold incubation buffer (250 mM sucrose, 5 mM Hepes-Tris, 2 mM EDTA, and 1 mM DTT), pH 7.8, bubbled vigorously with 95% O_2-5% CO_2. The lumimal surface was then firmly scraped with a glass microscope slide to harvest the trachea mucosa. The mucosal scrapings (5 g) were collected in 30 ml of ice-cold homogenization buffer (50 mM mannitol, 5 mM Hepes-Tris, 0.25 mM $MgCl_2$, and 1 mM DTT), pH 7.4. All subsequent steps were performed at 4°C. Following homogenization and centrifugation steps as detailed by Langridge-Smith et al. (1983) and Fong et al. (1988), suspensions containing the apical membranes were diluted in modified BBRS to achieve an approximate protein concentration of 5–7 mg/ml. Apical membranes were then stored at −80°C until use.

Protein content was measured by the Bio-Rad (Bio-Rad, Melville, NY) protein assay using bovine serum albumin as the standard. Alkaline phosphatase was chosen as the apical membrane marker and used to monitor consistency between apical membrane preparations. Alkaline phosphatase activity (μmol/h per mg protein) was determined following a modified procedure previously described (Weiser, 1973). Briefly, alkaline phosphatase activity was determined using Sigma 104 (Sigma Chemical Co., St. Louis, MO) phosphate substrate as the substrate and an assay medium containing 0.1 M Tris (pH 9.0), 2.5 mM $MgCl_2$, and 1% Triton-X-100. The reaction was initiated by the addition of a 200 μl aliquot of membrane suspension for a final volume of 850 μl including 50 μl of 1.32 mg/ml substrate. Following a 15 min incubation period at 37°C, 2.25 μl of ice-cold glycine buffer containing 133 mM glycine, 83 mM sodium carbonate, and 67 mM sodium chloride was added to stop any further reaction. A standard curve using *p*-nitrophenol was prepared to calculate alkaline phosphatase activity (μmol/h). The absorbances of samples and standards were recorded at 410 nm.

AMGZ, pH, and ionic strength effects on the lipid packing order of human erythrocyte ghosts and bovine trachea apical membranes

Erythrocyte ghosts at a final protein concentration of 100 μg/ml (Watala, 1988) and bovine

trachea apical membranes at a final concentration of 200 μg/ml (Schachter and Shinitzky, 1977; Brasitus and Keresztes, 1984; Worman et al., 1986) were added to the indicated AMGZ, pH or ionic strength solutions for a final volume of 2.5 ml. The erythrocyte ghosts and apical membrane suspensions were labeled with either DPH or TMA-DPH. DPH (1 mM) and TMA-DPH (0.5 mM) stock solutions were prepared fresh each day in tetrahydrofuran and dimethylformamide, respectively. DPH was added to each cuvette to obtain a final concentration of 2 μM. The probe was allowed to equilibrate with the membranes in the dark at room temperature for 30 min. TMA-DPH was added to each cuvette to obtain a final concentration of 1 μM. The probe only required a few minutes to become equilibrated with the membranes (Prendergast et al., 1981; Bronner et al., 1986).

Steady-state fluorescence anisotropy, r, of DPH and TMA-DPH labeled erythrocytes ghosts was measured with a SLM-AMINCO Subnanosecond Lifetime Fluorometer Model 4800c, equipped with a Zenith 158 computer for data analysis as previously detailed (Audus and Gordon, 1984, 1985; Audus et al., 1988, 1991).

Lifetime measurements

The excited state lifetimes (τ) of both DPH and TMA-DPH at varying AMGZ concentrations, pH values, and ionic strengths were calculated from phase and modulation measurements in erythrocyte ghosts as described previously (Lackowicz, 1983; Audus et al., 1985, 1988, 1991). The fluorophore probe's lifetimes were analyzed at both 18 and 30 MHz modulation frequencies. Dimethyl-POPOP, which has a known lifetime of approx. 1.45 ns, was utilized as the reference probe (Audus and Gordon, 1985; Audus et al., 1988, 1991).

Statistics

Results were presented as means $\pm$ the standard deviation (SD). Statistical analysis was performed with a one-way analysis of variance (ANOVA) using ABSTAT rel 6.00 (Anderson-Bell, Parker, CO). A value of $p < 0.05$ was considered statistically significant (Rosner, 1982).

Results

Fig. 1A shows AMGZ effects on erythrocyte hemolysis using a 50 mM NaCl as the hemolyzing 'control' solution. Significant hemolytic activity was observed at AMGZ concentrations greater than 1% ($p < 0.05$). At AMGZ concentrations 1.75–2.5%, a maximum of about 65% hemolysis, relative to the control hemolytic solution, was

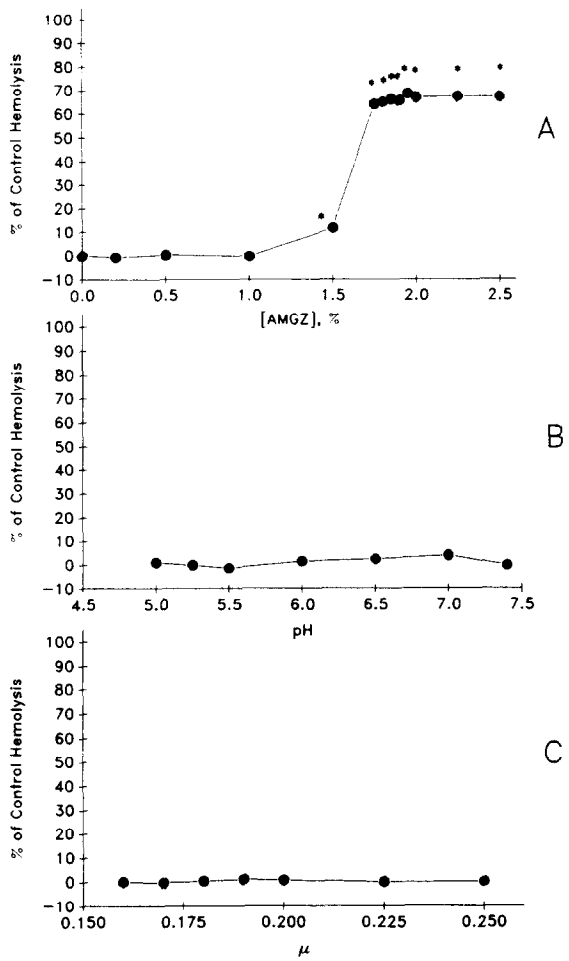

Fig. 1. Effects of selected ammonium glycyrrhizinate (AMGZ) concentrations (A), pH (B), and ionic strength (μ) (C) on erythrocyte hemolysis. Data points were calculated by dividing the mean degree of hemolysis in under indicated conditions by the mean control, hemolysis produced by the exposure to a hypotonic NaCl solution. Each data point represents a mean from an $n = 4 \pm$ SD (error bars are within the symbols in some cases). * Raw data statistically different from untreated control ($p < 0.05$).

observed. The concentration of AMGZ producing half-maximal hemolysis was approx. 1.5%. Fig. 1B and C shows the lack of lytic effect ($p > 0.05$) of varying pH and ionic strength on erythrocytes.

The effects of varying AMGZ concentration, pH, and ionic strength on erythrocyte ghost membrane order is illustrated in Fig. 2A–C. Under control conditions, pH 7.4, and ionic strength 0.16 μ, the average fluorescence anisotropy (r) values, were 0.301 ± 0.002 and 0.265 ± 0.003 for TMA-DPH and DPH labeled erythrocyte ghosts, respectively. Fig. 2A illustrates the concentration-dependent effects of AMGZ on the lipid order parameter of erythrocyte ghosts at 37°C. Significant decreases in r were observed in both TMA-DPH and DPH labeled erythrocyte ghosts at AMGZ concentrations greater than 0.5 and 1.0% ($p < 0.05$), respectively. The magnitude of the effects of AMGZ was significantly greater in the TMA-DPH (25% change) vs DPH (15% change) labeled erythrocyte ghosts ($p < 0.05$). Fig. 2B and C illustrates the lack of effects of varying pH and ionic strength on the lipid order parameter of erythrocyte ghosts at 37°C. Results from studies repeated under the same conditions as above at 25°C were qualitatively similar (data not shown).

Fluorescence lifetimes were monitored in erythrocyte ghosts labeled with either probe following exposure to AMGZ and other experimental conditions. Table 1 summarizes the calculated phase lifetimes (τ_Φ) for TMA-DPH in labeled erythrocyte ghosts in the presence of varying AMGZ concentrations, pH values, and ionic strengths. The calculated lifetimes were most sensitive to the presence of 1.0% AMGZ, 2% AMGZ, and pH 5.0, although none of the observed lifetime reductions were greater than 50%. Table 2 summarizes the calculated τ_Φ for DPH in labeled erythrocyte ghosts in the presence of varying AMGZ concentrations, pH values, and ionic strengths. Again, the calculated lifetimes were most sensitive to the presence of 1% AMGZ, 2% AMGZ, and pH 5.0 with none of the observed lifetime reductions greater than 50%. Paralleling changes in fluorescence anisotropy, the reductions in the fluorescence lifetimes of DPH

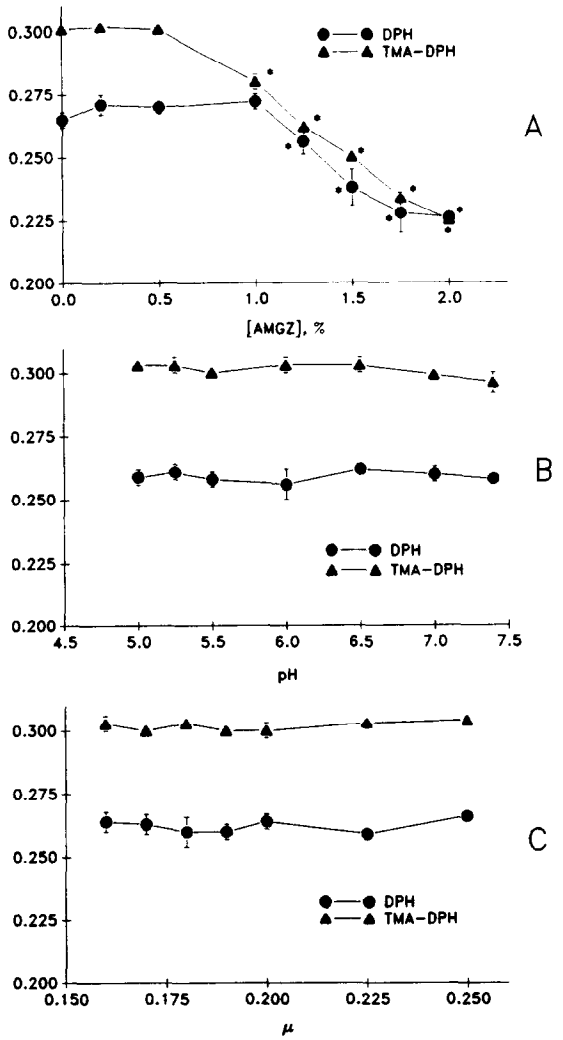

Fig. 2. Effects of selected ammonium glycyrrhizinate (AMGZ) concentrations (A), pH (B), and ionic strength (μ) (C) on the steady-state fluorescence anisotropy (r) of 1,6-diphenyl-1,3,5-hexatriene (DPH) or 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH) labeled erythrocyte ghosts at 37°C. Data points are the means ± SD for an $n = 3$ (error bars are within the symbols in some cases). * Raw data statistically different from untreated control ($p < 0.05$).

labeled domains were of a smaller magnitude as compared to TMA-DPH labeled domains.

The effects of varying AMGZ concentration, pH, and ionic strength on bovine trachea apical membrane structure is illustrated in Fig. 3A–C. Under control conditions, pH 7.4, and ionic

TABLE 1

Effects of ammonium glycyrrhizinate (AMGZ), pH, and ionic strength (μ) on the fluorescence phase lifetimes of 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH) in erythrocyte ghosts at 37°C

Treatment	Fluorescence lifetimes [a] (ns ± SD) [b]	
	30 MHz	18 MHz
Control	4.7 ± 0.1	5.2 ± 0.4
0.2% AMGZ	4.6 ± 0.1	5.6 ± 0.1
1% AMGZ	2.5 ± 0.1	2.5 ± 0.5
2.0% AMGZ	3.5 ± 0.2	3.9 ± 0.2
pH 5.00	3.7 ± 0.2	4.2 ± 0.3
$\mu = 0.17$	4.8 ± 0.2	6.0 ± 0.4
$\mu = 0.25$	4.5 ± 0.2	5.0 ± 0.5

[a] The phase lifetime (τ_Φ) of TMA-DPH was calculated from the expression $\tau_\Phi = 1/w$ tan Φ, where w was the angular frequency of excitation which was $2\pi \times$ modulation frequency (18 or 30 MHz) and Φ was the phase shift in degrees caused by a sinusoidally modulated emission from a fluorophore of lifetime, τ (Lackowicz, 1983).

[b] Data represent the means ± SD calculated from 5 alternately measured phase shifts and modulation changes of each sample measured relative to the reference, dimethyl-POPOP (Lackowicz, 1983).

TABLE 2

Effects of ammonium glycyrrhizinate (AMGZ), pH, and ionic strength (μ) on the fluorescence phase lifetimes of 1,6-diphenyl-1,3,5-hexatriene (DPH) in erythrocyte ghosts at 37°C

Treatment	Fluorescence lifetimes [a] (ns ± SD) [b]	
	30 MHz	18 MHz
Control	6.3 ± 0.3	6.7 ± 0.7
0.2% AMGZ	6.4 ± 0.2	7.5 ± 0.4
1% AMGZ	4.4 ± 0.2	5.3 ± 0.5
2.0% AMGZ	5.4 ± 0.2	6.3 ± 0.5
pH 5.00	5.0 ± 0.3	5.8 ± 0.7
$\mu = 0.17$	6.8 ± 0.2	7.7 ± 0.7
$\mu = 0.25$	6.4 ± 0.3	7.1 ± 0.5

[a] The phase lifetime (τ_Φ) of TMA-DPH was calculated from the expression $\tau_\Phi = 1/w$ tan Φ, where w was the angular frequency of excitation which was $2\pi \times$ modulation frequency (18 or 30 MHz) and Φ was the phase shift in degrees caused by a sinusoidally modulated emission from a fluorophore of lifetime, τ (Lackowicz, 1983).

[b] Data represent the means ± SD calculated from 5 alternately measured phase shifts and modulation changes of each sample measured relative to the reference, dimethyl-POPOP (Lackowicz, 1983).

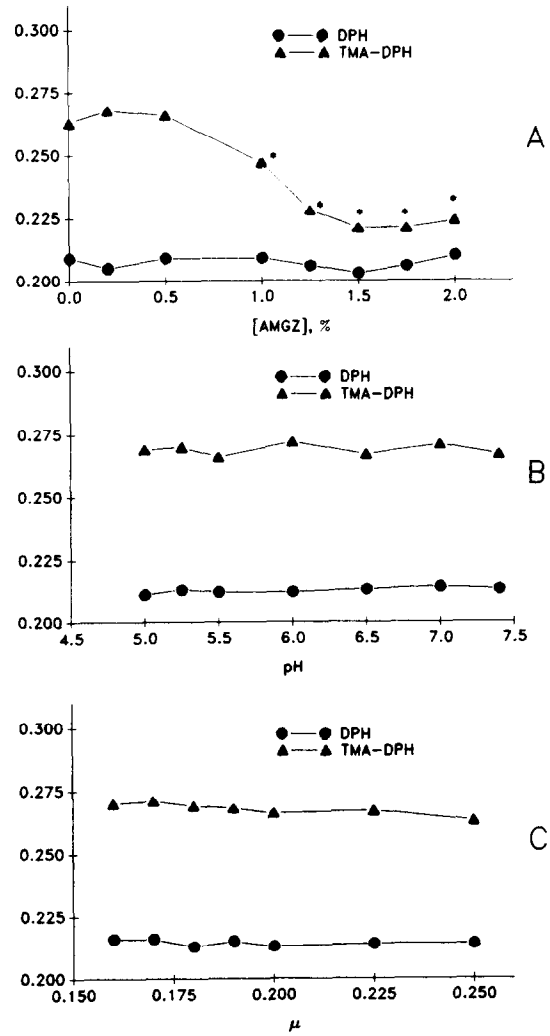

Fig. 3. Effects of selected ammonium glycyrrhizinate (AMGZ) concentrations (A), pH (B), and ionic strength (μ) (C) on the steady-state fluorescence anisotropy (r) of 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH) labeled bovine trachea apical membranes at 37°C. Data points are the means ± SD for an $n = 3$ (error bars are within the symbols in some cases). * Raw data statistically different from untreated control ($p < 0.05$).

strength 0.16 μ, the average r values were 0.263 ± 0.008 and 0.209 ± 0.001 for TMA-DPH and DPH labeled bovine trachea apical membranes, respectively. Fig. 2A illustrates the effect AMGZ on the r values for TMA-DPH in labeled bovine trachea apical membranes at AMGZ concentrations greater than 0.5% ($p < 0.05$). In contrast to

the erythrocyte ghost, a significant change in r was not evident in DPH labeled bovine trachea apical membranes at varying AMGZ concentrations ($p > 0.05$). Fig. 2B and C again illustrates the lack of effects of pH and ionic strength on the r of DPH and TMA-DPH in labeled bovine trachea apical membranes at 37°C. Under similar experimental conditions at 25°C qualitatively similar results were observed (data not shown).

Discussion

Hemolysis of erythrocytes has been previously employed to determine the membrane perturbation of potential absorption promoters. The hemolytic activity of glycyrrhetinic acid and derivatives was determined by Mishima et al. (1989) to be less or similar to that of sodium glycocholate, thus suggesting that these compounds were just as likely to cause acute irritation to the nasal mucosa. A correlation between erythrocyte hemolysis and absorption-promoting effects has also been reported for non-ionic surfactants, saponin, and the anionic surfactant, sodium lauryl sulfate (Hirai et al., 1981). In this study, significant hemolysis was observed at AMGZ concentrations greater than 1%. The pH of a 2% AMGZ solution in BBRS is 5.25 and the ionic strength 0.17 μ. These changes could also potentially contribute to the hemolytic activity of AMGZ. We observed that hemolysis did not occur over a pH range of 5.0–7.4 and a range of ionic strength of 0.16–0.25 μ. The latter conditions may more likely result in some degree of crenation. Therefore, these results suggested that hemolysis and the permeability enhancing properties may be due in part to a direct AMGZ effect on the membrane structure and not due to a pH or an ionic strength effects.

The fluorophores chosen to characterize the effect of AMGZ on the molecular packing order of membrane lipids in this study were DPH and TMA-DPH. DPH localizes within the hydrophobic core of lipid bilayers, and measures the lipid-order or the average molecular packing of deeper lipid or hydrophobic regions of membranes. TMA-DPH, a cationized probe, localizes at the lipid-water interface, and measures the lipid-order or the average molecular packing at the surface of membranes. The use of DPH and TMA-DPH to determine the lipid order of membranes has been previously established (Lakowicz et al., 1979; Engel and Prendergast, 1981; Prendergast et al., 1981; Van Blitterswijk et al., 1981; Pottel et al., 1983). Fluorescence anisotropy techniques have been also previously employed to specifically assess the fluidity of blood cell membranes (Klausner et al., 1980; Kitagawa et al., 1991; Tangorra et al., 1991) and of bovine trachea apical membranes (Worman et al., 1986).

The changes in the molecular packing order for fluorophore-labeled lipid domains of the erythrocyte ghost membranes, as reflected by changes in r of DPH and TMA-DPH, decreased with increasing concentrations of AMGZ. Changes in pH and ionic strength corresponding to the AMGZ concentration range examined, did not alter the molecular packing order of the erythrocyte ghosts. Apparent from this study also was the more dramatic effect of AMGZ at the surface of the labeled membranes at concentrations greater than 0.5%. Higher concentrations, > 1%, were required to alter the domains labeled with DPH and corresponded more closely with those AMGZ concentrations that caused hemolysis.

Fluorescence lifetime measurements are generally required to properly interpret fluorescence anisotropy changes for a fluorophore (Lackowicz, 1983; Sklar, 1984). In this study, fluorescence lifetime determinations were required to evaluate potential contributions of direct interactions between AMGZ and the fluorophore (DPH or TMA-DPH) in the labeled membranes. The fluorescence lifetime measurements under control conditions, pH 7.4, and ionic strength of 0.16 μ, were typical of those reported in other cell types and for model membrane systems (Klausner et al., 1980; Cranney et al., 1983; Audus and Gordon, 1984, 1985; Audus et al., 1991). For a decrease in the fluorescence lifetime to significantly affect the anisotropy of DPH and TMA-DPH, and hence, the lipid packing order, at least a 50% reduction in the lifetime would have to be occur (Sklar, 1984). Therefore, for erythrocyte ghosts treated with AMGZ a significant decrease in the

lifetimes of DPH and TMA-DPH did not correlate with the decrease in the fluorescence anisotropy with one exception; the exposure of TMA-DPH labeled erthrocyte ghost membranes to 1% AMGZ. Not coincidently perhaps, this corresponds to the concentration after which significant hemolysis occurs and may relate to subtle surface changes preceding membrane disruption. The greater sensitivity of the fluorescence lifetime of TMA-DPH as compared with DPH may be due to TMA-DPH's position at the surface of the membrane thus making it more susceptible to quenching effects as observed by others (Prendergast et al., 1981). The lifetime of DPH probes decreases on exposure to a more hydrophilic environment and are nonfluorescent in an aqueous solution (Klausner, 1980; Lackowicz, 1983). Consequently, the changes in fluorescence lifetime of particularly, TMA-DPH, might also be interpreted as a change in the membrane domain of the probe such that exposure of the probe to a more aqueous environment occurs in the presence of AMGZ. Conservatively interpreted, the changes in fluorescence anisotropy and the fluorescence lifetimes probably reflect significant contributions of AMGZ effects on the membrane and of potentially direct AMGZ:probe interactions. Moreover, the lifetime studies confirmed the dominant AMGZ effects on the lipid-water interface of the erythrocyte ghost membrane.

The cellular composition of the nasal, tracheal, and upper respiratory system are similar. The apical membranes of these tissues are the initial sites of interactions for absorption promoters such as AMGZ. Attempts were made to isolate the apical membranes of ovine nasal mucosal, the airway epithelium with which previous studies had been conducted (Reardon et al., 1993). However, the availability of tissue and resulting yields of apical membrane preparations were inadequate for these studies. Alternatively, apical epithelial cell membranes from bovine trachea were isolated in sufficient quantities to carry out the fluorescence anisotropy studies. Isolation of apical membranes was confirmed by the enrichment of alkaline phosphatase relative to the basolateral membrane isolations (Langridge-Smith et al., 1983; Fong et al., 1988).

Under control conditions, pH 7.4, and ionic strength 0.16 μ, the fluorescence anisotropy measures of TMA-DPH and DPH in labeled erythrocyte ghosts were significantly higher than those for bovine trachea apical membranes. This observation indicated that the membrane order was greater in erythrocyte ghosts as compared with bovine trachea apical membranes and may correspond to higher protein/lipid ratios (w/w) in the tissues. For human erythrocyte ghosts the ratio is 1.14 (Guidotti, 1972) compared to bovine trachea apical membranes which is 0.60 (Worman et al., 1986). A higher protein/lipid ratio (w/w), similar to addition of cholesterol to a membrane, has been associated with an ordering effect on lipid packing (i.e., decreased membrane fluidity) in biomembranes (Fraley et al., 1978; Heyn, 1979; Brasitus and Schachter, 1980; Kajii et al., 1986). Alternatively, the lipid composition of the membranes of the individual cell types may differ and result in differences in molecular packing order (Klausner, 1980).

The differences in tracheal epithelial cell apical membrane composition, relative to the erythrocyte membrane, may provide a possible explanation for the observation that AMGZ concentrations greater than 0.5% altered membrane packing order only in TMA-DPH labeled superficial domains but had no signficant effect on deep membrane domains labeled with DPH. Similar observations have been made in model membrane systems where those membranes with lower lipid packing order, i.e., being more fluid or flexible, were more resistant to membrane perturbing drugs (Audus and Gordon, 1985). Perhaps this may be manifested in previous tissue studies (Reardon et al., 1993) where the nasal mucosal cells appear to resist lysing in the presence of AMGZ concentrations that enhanced permeability of the tissue to low molecular weight markers. Neither corresponding pH nor ionic strength variation altered the r parameter in the TMA-DPH and DPH labeled membrane domains, consistent with the erythrocyte ghost membranes studies.

The present study addressed the relationship between AMGZ's absorption promoting effect and its effect on membrane structural integrity by studying the hemolytic activity and changes in

membrane lipid order. AMGZ produced significant hemolytic activity and evidence for a decrease in the lipid order at the surface of bovine trachea apical membranes and erythrocyte ghosts at concentrations greater than 0.5%. However, surface probe (TMA-DPH) effects in either membrane type were observed at lower AMGZ concentrations than hemolysis and core-probe (DPH) effects in the erythrocyte. These results paralleled previous results in which a 4–5 fold increase in permeation of low molecular weight markers, mannitol and Lucifer yellow, across ovine nasal mucosa in vitro was observed in the presence of similar concentrations of AMGZ (Reardon et al., 1993). By contrast, the hemolytic activity and the lipid order of bovine trachea apical membranes and erythrocyte ghosts were not affected by varied pH or ionic strength. These results suggested that the increase in the permeability of the ovine nasal mucosa to mannitol and lucifer yellow in the presence of AMGZ may be due, at least in part, to the perturbation or modification of the structural integrity of the nasal mucosal caused by a direct AMGZ effect on membrane components of the lipid:water interface and not by a pH or an ionic strength effect.

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