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Interaction of 1-dodecylazacycloheptan-2-one (Azone) with erythrocyte membrane

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Summary

The action of 1-dodecylazacycloheptan-2-one (Azone) known as a skin permeation enhancer and its methyl analogue (1-methylazacycloheptan-2-one) on erythrocyte membrane was studied Azone caused hemolysis, but the methyl analogue did not. Azone afforded dose-dependently a large membrane lesion in erythrocytes. The release of membrane fragments containing phospholipids was also detected under the conditions of the lesion. Azone had an ability to penetrate into a liposomal membrane composed of dipalmitoylphosphatidylcholine, but the methyl analogue did not Thus, the following mechanism was proposed to explain the permeability-increasing action of Azone The agent entered into erythrocyte membrane and made the membrane structure unstable, resulting in both the release of membrane fragments outside cells and the enhancement of permeability.

Introduction

1-Dodecylazacycloheptan-2-one (Azone) has been reported as a new penetration enhancer for the percutaneous delivery of a number of drugs (Stoughton and McClure, 1983; Barry, 1987). The penetration-enhancing effect of Azone is believed to be due to its increasing the fluidity of the intercellular lipid bilayers of the stratum corneum. It has been reported that Azone abolishes the endothermic peaks in differential scanning calorimetry attributable to the phase transition of the lipids of the stratum corneum (Goodman and Barry, 1985). Quite recently, Beastall et al. (1988) have reported that Azone decreases the phase transition temperature of an artificial liposomal membrane composed of dipalmitoylphosphatidylcholine (DPPC). These indicate that Azone is able to penetrate into lipid bilayers to increase the fluidity of membranes. Such an increase in the fluidity may be important to reduce the diffusional barrier against drugs in the stratum corneum. Kurosaki et al. (1989), co-workers in the present study, have also reported the enhancing effect of Azone on the permeability of keratinized oral-mucosa and clarified the enhancing characteristics of Azone as follows: (1) Azone acts directly on the stratum corneum of the keratinized oral-

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mucosa where the major barrier function exists; and (2) Azone molecules penetrate into intercellular lipid layers of the stratum corneum and increase the fluidity of the lipid layers.

We were interested in whether Azone could enhance the permeability of other biological membranes than that of skin. In this work, we examined the action of Azone and its methyl analogue (1-methylazacycloheptan-2-one) on human erythrocyte membrane. Significant differences exist in membrane structure between the stratum corneum and erythrocytes: e.g. the multilamellar nature of the stratum corneum vs the unilamellar nature of red cells; the different lipid compositions; the presence of a cytoskeleton underneath the lipid bilayer of red cell membrane. However, the present study will also help to clarify the mechanism of permeability enhancement of skin by Azone. It was found that Azone increased the permeability of erythrocytes, but the methyl analogue did not. We determined the size of the lesion formed in the erythrocyte membrane, and found that Azone caused a larger lesion with an increase in the concentration. Furthermore, the release of membrane fragments containing phospholipids was confirmed under the conditions of membrane lesion. The present study shows that Azone has the ability to release membrane fragments out of cells and brings about an increase in the permeability of erythrocytes simultaneously.

Materials and Methods

Chemicals

Azone was kindly supplied by Nelson-Sumisho Co., Tokyo. 1-Methylazacycloheptan-2-one and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Tokyo Kasei Kogyo Co., Tokyo; DPPC from Sigma, St. Louis, MO; D-mannitol, sucrose, raffinose and polyethylene glycols (PEGs) whose average molecular weights were 400, 600, 1000 and 2000 from Wako Pure Chemical Industries, Osaka. Other chemicals used were all of analytical reagent grade.

Erythrocytes

Human erythrocytes were used. Cells were washed twice with an isotonic buffer solution comprising 0.15 M NaCl and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES)–NaOH (pH 7.4) and suspended in this buffer solution at concentration of 5% (v/v).

Measurement of hemolysis

Erythrocytes were suspended in 2 ml of 0.15 M NaCl and 5 mM HEPES–NaOH (pH 7.4) at the final concentration of 0.5% (v/v). Then, Azone dissolved in ethanol solution was added and incubated at 37 °C for 30 min. The final amount of ethanol did not exceed 1% (v/v). After cells were centrifuged out, the degree of hemolysis was determined by measuring the absorbance of the supernatant at 540 nm. The total amount of hemoglobin was estimated after erythrocytes were lysed by adding water.

Osmotic protection experiments

Erythrocytes (0.5% (v/v)) were suspended in an assay solution of 0.135 M NaCl, 5 mM HEPES–NaOH (pH 7.4) and 30 mM of one of the following substances: D-mannitol; sucrose; raffinose; PEGs 400, 600, 1000 and 2000. Then, Azone was added and hemolysis was determined after incubation for 30 min at 37 °C. The following molecular diameters of substances were used for estimation (Katsu et al., 1988): mannitol, 0.7 nm; sucrose, 0.9 nm; raffinose, 1.1 nm; PEGs 400, 600, 1000 and 2000, 1.2 nm, 1.6 nm, 2.0 nm and 2.9 nm, respectively. The efflux of K^+ from erythrocytes was measured with a K^+ ion-selective electrode as reported previously (Katsu et al., 1986).

Phosphorus assay

After erythrocytes (0.5% (v/v)) were incubated with Azone at 37 °C for 30 min in a buffer solution comprising 0.15 M NaCl and 5 mM Hepes–NaOH (pH 7.4), cells were centrifuged at $14{,}000 \times g$ for 1 min (Billington and Coleman, 1978). Lipid extracts (Bligh and Dyer, 1959) from the supernatant were assayed for phospholipid phosphorus (Galliard et al., 1965). The total amount of phospholipids contained in erythrocytes (0.5% (v/v)) was 30 μM in DPPC equivalent.

Fluorescence polarization

Agent-induced changes in the phase transition temperature of DPPC liposomes were measured

by the fluorescence polarization technique as reported previously (Katsu et al., 1986). DPH (1 mol% of DPPC) was used as a fluorescence probe. DPPC containing DPH was swollen in a buffer solution of 0.15 M NaCl and 5 mM HEPES–NaOH (pH 7.4) at 55 °C to prepare multilamellar liposomes. A small aliquot of the liposomes was pipetted and suspended in the same buffer at the final concentration of 100 μM of lipid. Then the agent was added to this suspension, and the suspension was briefly sonicated (for about 10 s at 55 °C) to penetrate the agent deeply into the multilamellar liposomes. Fluorescence polarization was measured by using a Hitachi MPF-4 fluorospectrophotometer equipped with polarizers and thermoregulated cells. The degree of polarization (P) was calculated according to the following equation:

$$P = \frac{I_{VV} - C_f I_{VH}}{I_{VV} + C_f I_{VH}}$$

where I is the fluorescence intensity, and subscripts V and H refer respectively to the vertical and horizontal orientations of the excitation (first) and emission (second) polarizers. C_f ($= I_{VH}/I_{HH}$) is a correction factor (Azumi and McGlynn, 1962). The temperature in the cell was measured with a thermister. A small amount of ethanol used as the solvent of agents did not affect the phase transition temperature of DPPC at all.

Results

At first, we investigated the effects of Azone and its methyl analogue on the phase transition temperature of DPPC (Fig. 1). Azone lowered the phase transition temperature as reported previously (Beastall et al., 1988), but the methyl analogue did not produce any effect at all. This fact indicates that Azone penetrates into the lipid bilayer to increase the movement of the acyl chains, while the methyl analogue cannot enter into the bilayer, probably due to the lack of a lipophilic dodecyl group. We have recently found that, when drugs are able to penetrate into the gel phase of a zwitterionic lipid such as DPPC and decrease the

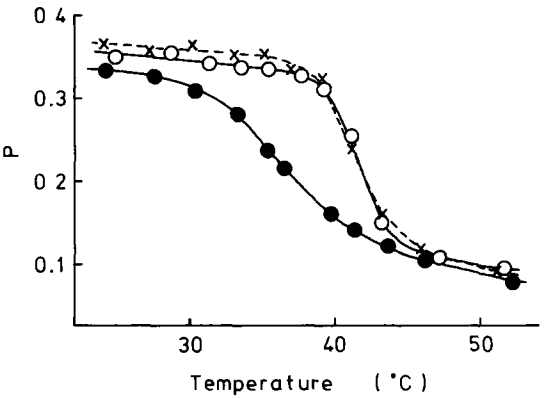

Fig. 1 Changes in the degree of polarization (P) of DPPC liposomes ×, DPPC liposomes alone (100 μM), ●, after addition of Azone (50 μM); ○, after addition of the methyl analogue (200 μM).

phase transition temperature, they increase the permeability of various kinds of the cytoplasmic membrane of cells (Katsu et al., 1987). It is probable that, if drugs have a strong penetration ability into even the gel phase of DPPC membrane, they can also enter into the biological membrane mainly composed of zwitterionic phospholipids to increase the permeability. In accordance with this view, incubation of red cells with Azone induced hemolysis markedly, but the methyl analogue of Azone caused little hemolysis even at the concentration of 200 μM (Fig. 2).

Then, we determined the size of Azone-induced membrane lesion in erythrocytes by means of osmotic protection experiments (Katsu et al., 1988). This experiment is based on the fact that, if extra-added solute cannot intrude into erythrocytes through membrane lesion and the osmotic pressure of intracellular hemoglobin is balanced with that of the solute, hemolysis is not provoked. Thus, the size of lesion can be determined by examining whether hemolysis is protected or not. Fig. 3 shows the protection of hemolysis induced by Azone (50 μM). In Fig. 3, the degree of hemolysis was plotted as a function of the diameters of the protectants. Although sugars and PEGs of lower molecular weights did not protect hemolysis at all, PEGs 1000 and 2000 protected hemolysis. This means that the size of membrane lesion induced by Azone (50 μM) was about 2.0 nm,

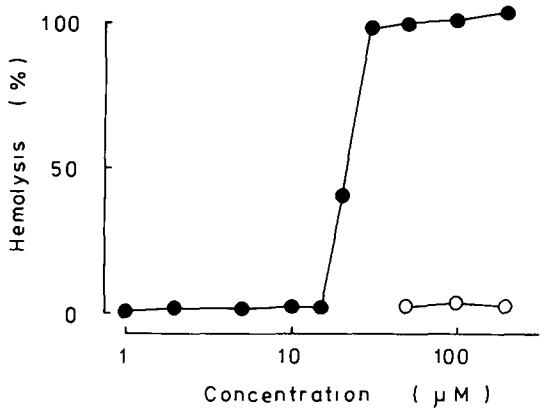

Fig 2 Hemolysis induced by Azone and the methyl analogue ●, Azone, ○, the methyl analogue Erythrocytes (0 5% (v/v)) were suspended in 0 15 M NaCl and 5 mM HEPES–NaOH (pH 7 4). Hemolysis was determined after cells were incubated with the agent at 37 °C for 30 min

corresponding to the diameter of PEG 1000. Then we tried the osmotic protection experiments at various concentrations of Azone (Fig. 4). The dose–response curves of hemolysis in the presence of mannitol, sucrose and PEG 400 were almost

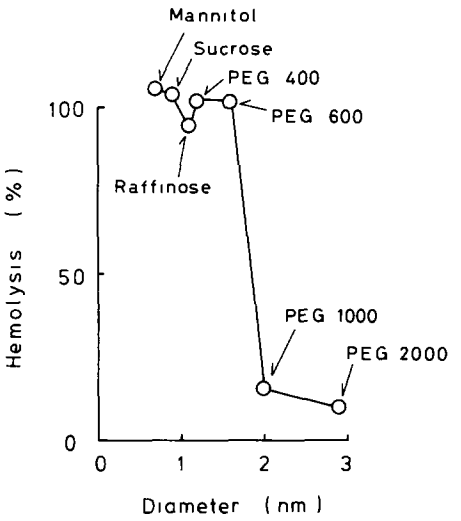

Fig. 3 Hemolysis induced by Azone (50 μM) in the presence of various colloid-osmotic protectants Erythrocytes (0.5% (v/v)) were suspended in 0.135 M NaCl, 5 mM HEPES-NaOH (pH 7.4) and 30 mM protectant Hemolysis was determined after cells were incubated with Azone at 37 °C for 30 min. The diameters of protectants are described in Materials and Methods.

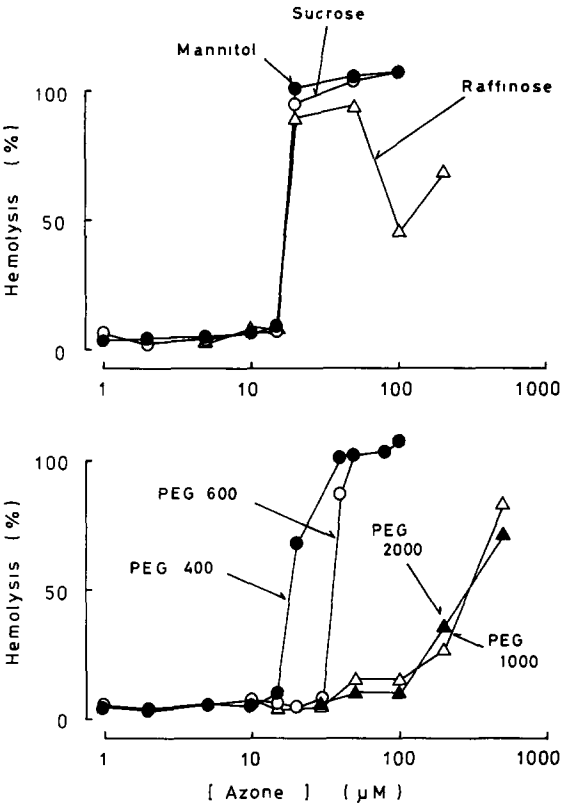

Fig. 4 Protection of hemolysis induced at various concentrations of Azone. Assay conditions were the same as in Fig 3, except that the concentration of Azone was changed.

similar and the threshold concentrations of Azone causing hemolysis were around 15 μM in all cases. This indicates that 15 μM of Azone produced the lesion of 1.2 nm corresponding to the diameter of PEG 400 which had the largest diameter among them. In a similar way, we could estimate that Azone formed the lesion of 1.6 nm (diameter of PEG 600) at 30 μM, and 2.9 nm (diameter of PEG 2000) at 100 μM. Here, we should mention hemolysis in the presence of raffinose. Although hemolysis in the presence of raffinose showed almost the same behaviour as in the cases of other sugars and PEG 400 at lower concentrations of Azone, hemolysis was greatly suppressed at around 100 μM of Azone. Why hemolysis decreased at this concentration cannot be explained at present. However, such a decrease in hemolysis did not disturb the determination of the size of membrane

lesion, because the threshold of hemolysis was clearly observed at a lower concentration. Then, a question was directed to the results of hemolysis in the presence of PEGs 1000 and 2000. Also, in these cases, decreases in hemolysis might be due to the inhibition of Azone action. In this respect, it is worthwhile to measure changes in the K^+ permeability of cells in the presence of protectants (Yamanaka et al., 1987). If Azone causes membrane lesion, the efflux of K^+ from cells must be observed. Indeed, the efflux of K^+ was observed even when the presence of PEG 1000 or 2000 inhibited hemolysis, indicating that the protectants did not affect the formation of membrane lesion by Azone. From these data, we could depict a change in the size of membrane lesion depending on the concentration of Azone as in Fig. 5. It was found that the size of lesion increased with increases in the concentration of Azone.

We presumed that Azone might release the membrane fragments from erythrocytes; as the amount of Azone penetrated into the erythrocyte membrane increased, the membrane was seriously broken, resulting in a large membrane lesion. To confirm such fragment release, phospholipids existing in the supernatant were assayed. Fig. 6 shows the release of phospholipids as a function of the concentration of Azone. It should be emphasized that the release began to increase at around 15 μM of Azone, where the initial membrane lesion was induced. Furthermore, the release of phospholipid increased markedly with an increase in the concentration of Azone, reaching about 40% at the concentrations above 30 μM.

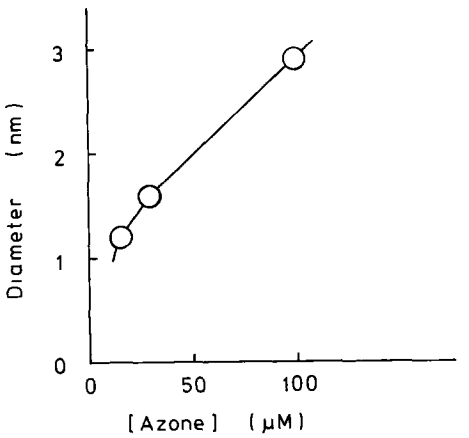

Fig 5. Plot of the size of membrane lesion vs the concentration of Azone

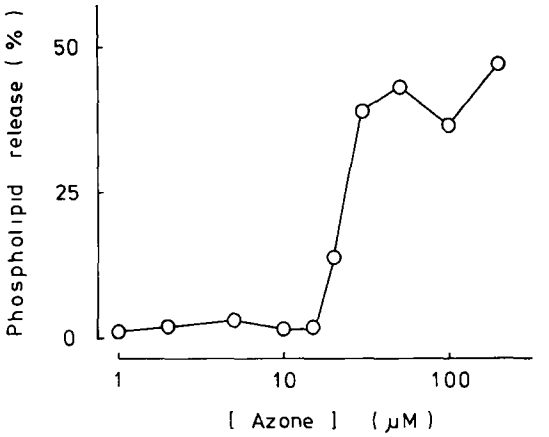

Fig 6. Plot of the release of phospholipids vs the concentration of Azone

Discussion

So far, many workers have investigated the drug–membrane interaction; particularly, attention has been paid to the effects of drugs on membrane fluidity and membrane permeability (Juliano, 1983). Papahadjopoulos and co-workers (1975) have classified some of these effects. One type of interaction involves the penetration of substances into lipid bilayer to stimulate the movement of acyl chains of phospholipid molecules and decreases the phase transition temperature as in the present case of Azone. The typical examples were obtained with basic myelin protein and cytochrome C. These basic peptides have an appropriate lipophilic character and interact preferentially with negatively charged phospholipids to decrease their phase transition temperatures; however, they did not affect at all the phase transition temperature of zwitterionic phospholipid DPPC. Among many amphiphilic drugs, gramicidin S is known to decrease the phase transition temperature of DPPC (Katsu et al., 1986). Investigation of the structure–activity relationship of gramicidin S analogues revealed that the analogues having the ability to decrease the phase

transition temperature of DPPC were able to increase the permeability of various kinds of cells (Katsu et al., 1987). Because Beastall et al. (1988) showed that Azone decreased the phase transition temperature of DPPC, we expected that Azone might enhance the permeability of red cells.

The present results are summarized as follows: (1) Azone decreased the phase transition temperature of DPPC, but the methyl analogue did not; (2) Azone caused hemolysis, but the methyl analogue did not; (3) Azone formed larger membrane lesions when its concentration increased; and (4) the release of membrane fragments containing phospholipids was observed under the conditions of membrane lesions. From these results, the following action mechanism of Azone on erythrocyte membrane was suggested. The dodecyl group of Azone molecule penetrated deeply into membrane, while the amide group might preferentially be located near the hydrophilic region of lipids because of its somewhat hydrophilic nature (Barry, 1987). Thus, a bulky ε-caprolactam ring thrust up in the vicinity of the polar head of lipids and deformed greatly the lipid packing. The large accumulation of the agent resulted in both the liberation of some membrane fragments out of cells and the enhancement of permeability. Similar effects were also observed recently with the interaction between some amphiphilic peptides and erythrocyte membrane (Katsu et al., 1988). However, further studies are needed to clarify whether Azone acts on the lipid bilayer of the stratum corneum in a similar way.

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