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Roles of integral protein in membrane permeabilization by amphidinols

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Abstract

Amphidinols (AMs) are a group of dinoflagellate metabolites with potent antifungal activity. As is the case with polyene macrolide antibiotics, the mode of action of AMs is accounted for by direct interaction with lipid bilayers, which leads to formation of pores or lesions in biomembranes. However, it was revealed that AMs induce hemolysis with significantly lower concentrations than those necessary to permeabilize artificial liposomes, suggesting that a certain factor(s) in erythrocyte membrane potentiates AM activity. Glycophorin A (GpA), a major erythrocyte protein, was chosen as a model protein to investigate interaction between peptides and AMs such as AM2, AM3 and AM6 by using SDS-PAGE, surface plasmon resonance, and fluorescent-dye leakages from GpA-reconstituted liposomes. The results unambiguously demonstrated that AMs have an affinity to the transmembrane domain of GpA, and their membrane-permeabilizing activity is significantly potentiated by GpA. Surface plasmon resonance experiments revealed that their interaction has a dissociation constant of the order of 10 μM, which is significantly larger than efficacious concentrations of hemolysis by AMs. These results imply that the potentiation action by GpA or membrane integral peptides may be due to a higher affinity of AMs to protein-containing membranes than that to pure lipid bilayers.

Keywords: Antifungal; Amphidinol; Hemolysis; Glycophorin A

1. Introduction

Amphidinols (AMs) are a class of dinoflagellate metabolites isolated from *Amphidinium klebsii* [1], and possess very potent antifungal activity [2–4]. Several structurally related analogues, luteophanols, lingshuiols and karatungiols, were reported from the same genus of dinoflagellate [5–7]. The skeletal structure of AMs is characterized by a common portion, which comprises a linear polyhydroxyl moiety, two tetrahydropyran rings, and a skipped polyene chain of fourteen or sixteen carbon atoms (Fig. 1). Their structures are reminiscent of polyene macrolide antibiotics because both of them encompass polyolefinic and polyhydroxyl chains. However, their molecular architectures are substantially different in flexibility; molecular modeling studies have clearly demonstrated that amphotericin B, the best-known polyene antibiotic, has a rigid macrolactone ring while AMs take highly dispersed conformers [8]. Therefore, AMs may be categorized as an unknown type of antifungals with respect to chemical structures and mode of actions, hence stimulating research on their mechanism [2,4,8–11], which may lead to developments of new fungicidal drugs.

Around fourteen amphidinols have been isolated so far. Among those, AM3 [12] reveals most potent hemolytic activity, which significantly exceeds those of other major constituents [8]. AMs increase the permeability of the biological membranes by directly interacting with a lipid bilayer [9], which is thought to be responsible for their powerful antifungal activity. In the previous report [8], we proposed a molecular mode of action on

Abbreviations: PC, phosphatidylcholine; GpA, glycophorin A; AM, amphidinol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EDTA, ethylenediamine tetraacetic acid; NHS, *N*-hydroxysuccinimide; EDC, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride; CTAB, Cetyltrimethylammonium bromide; SUV, small unilamellar vesicles; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; PBS, phosphate buffered saline; BSA, bovine serum albumin; GpA-TM, glycophorin A transmembrane peptide; NIES, National Institute of Environmental Studies

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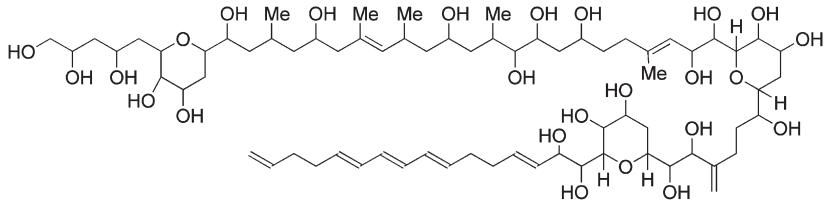
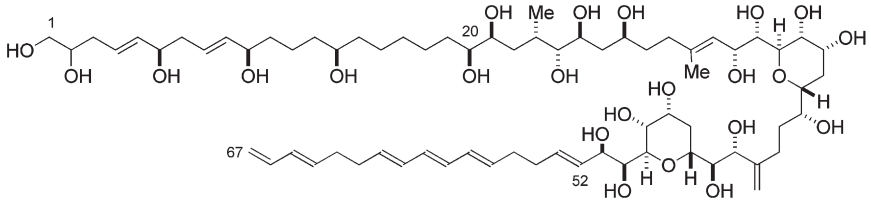
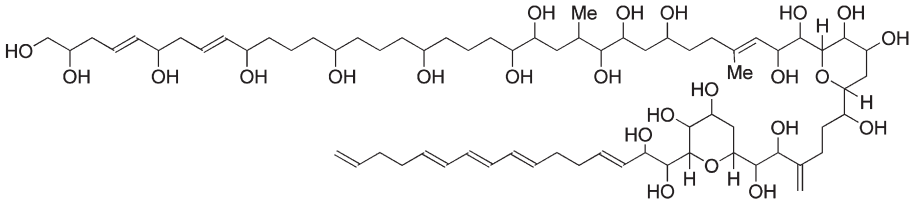


AM2



AM3



AM6

	R_1	R_2
AM7	OSO_3Na	
dsAM7	OH	
AM14	OSO_3Na	
AM15	OH	

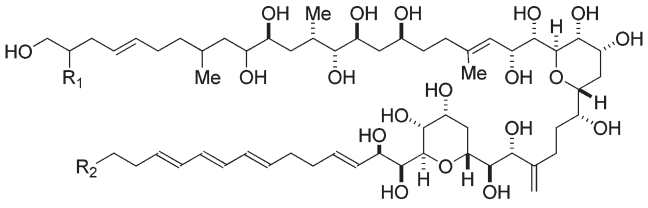


Fig. 1. Structures of amphidinols (AMs).

the basis of the conformation of AM3 in sodium dodecyl sulfate (SDS) micelles; AM3 binds to bilayer membrane chiefly with the polyene part (C52–C67 of AM3) while the central hydrophilic region (C20–C51 of AM3) takes a hairpin-shaped conformation, which is stabilized by intramolecular hydrogen bonds. We also reported that AMs are more efficacious in hemolytic activity than in membrane permeabilization with artificial liposomes [8,9]. In the case of polyene macrolide antibiotics, sterols play an essential role in the selective toxicity; e.g., amphotericin B may be the most investigated drug, whose selective toxicity to fungi is usually accounted for by the higher efficacy with ergosterol-containing bilayers than cholesterol-containing ones. However, the activity of AMs can be stimulated either by cholesterol or by ergosterol although membrane sterol is necessary for the activity [11,13]. These characteristic features prompted us to examine constituents of biological membranes that potentiate the AM activity.

Glycophorin A (GpA), a major membrane integral protein of erythrocyte, is a single polypeptide chain of 131 amino acids [14], which is heavily glycosylated with 16 oligosaccharide chains [15]. It shows strong self-association, and resides as dimers in bilayer membranes [16]. GpA plays an interesting role as a receptor for some peptide toxins such as alpha-hemolysin [17] and aerolysin [18,19]. It is reported [17] that the hemolysin binds to glycophorin in erythrocytes and this binding is abolished by trypsinization of membrane proteins, and that GpA-reconstituted liposomes significantly increase their sensitivity to the hemolysin

action. In addition, aerolysin disrupts liposomes at concentrations 2–3 orders of magnitude higher (less efficacious) than erythrocytes [18,19].

In this study we carried out hemolysis assays and fluorescent-dye leakage experiments using artificial liposomes in order to compare the AM-induced activity between biological and artificial membrane systems. We also investigated interactions between AMs and GpA using SDS-PAGE and SPR measurements.

2. Materials and methods

2.1. Materials

Amphidinols were isolated from the marine dinoflagellate *A. klebsii*, which had been separated from seawater in Aburatsubo-Bay, Kanagawa, Japan, and deposited in the National Institute of Environmental Studies (NIES 613). The culture medium was artificial seawater (Marin Art Hi, Tomita Pharmaceutical, 3% w/v) enriched with ES-1 supplement. The unialgal culture was grown in a 3-L glass flask containing 2 L of 80% seawater enriched with GSe supplements. Extract and purification of amphidinols were carried out as previously reported [20].

Calcein (Fluorexone) and egg yolk lecithin (phospholipid purity, 70%) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Cholesterol, ergosterol, human glycophorin A (from blood type MM), monoclonal anti-glycophorin A (α), bovine serum albumin (BSA), avidin, streptavidin, trypsin and α (2→3,6,8,5) neuraminidase from *Arthrobacer ureafaciens* were obtained from Sigma-Aldrich (St. Louis, MO). Glycophorin A transmembrane domain (GpA-TM: EPEITLIIFGVMAGVIGTILLISYGIRRL) was chemically synthesized as previously reported [21]. Silver Staining Kit Protein® was purchased from Amersham Biosciences (Piscataway, NJ). All the other chemicals were standard and analytical quality reagents.

2.2. Hemolysis assays

Human red blood cells suspended in 3.13 % (w/v) sodium citrate were immediately separated from the plasma by centrifugation at 1000 ×g for 5 min. Sedimented cells were washed three times with PBS buffer containing 137 mM NaCl, 2.68 mM KCl, 8.10 mM Na_2HPO_4, and 1.47 mM KH_2PO_4 (pH 7.4) to be 1% hematocrit. Amphidinol in 10 μL of MeOH was preincubated with or without various concentrations of GpA (10 μL), and added to the red blood cell suspension (180 μL) and incubated for 6 h at 37 °C. After centrifugation, the supernatant was subjected to colorimetric measurements at 450 nm on Emax® Precision Microplate Reader (Molecular Devices Corporation, Sunnyvale, CA). The complete lyses were obtained when the red blood cells were exposed to water. The percentage of hemoglobin released from erythrocytes was calculated. In order to examine blockade of AMs' interaction with GpA by anti GpA monoclonal antibody, 180 μL of red blood cell suspension in 1% hematocrit in PBS was incubated for 1 h at room temperature with 10 μL of various concentrations of the antibody dissolved in PBS. The resultant erythrocytes were washed three times with the same PBS buffer, and suspended in 190 μL of PBS for the hemolysis assays described in the above. Enzymatic digestion in advance to the hemolysis assays was carried out as follows: one hundred seventy microliters of red blood cells at 1% hematocrit in PBS was incubated with neuraminidase for 3 h at 37 °C. The cell suspension was washed three times with PBS, and was suspended in 190 μL of PBS buffer. The hemolytic activity of AM was tested as described in the above.

Determination of lipid concentration in erythrocyte membrane was carried out as follows. Two milliliters of erythrocyte membranes in PBS were centrifuged at 1000 ×g for 5 min. Sedimented cells were washed three times with PBS buffer. The PBS was replaced with 10 mL of water to cause complete hemolysis. The mixture was centrifuged at 15,000 ×g for 1 h. The sedimented membrane lipids were washed three times and subjected to a phospholipid C-Test® (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

2.3. Calcein leakage from liposomes

Large unilamellar vesicle (LUV) liposomes were prepared as follows. Lipid (20 mg) with or without sterol was dissolved in 3 mL of $CHCl_3$. After evaporation of $CHCl_3$ at 30 °C under vacuum for 2 h, it was dried in *vacuo* overnight. The lipid film was suspended in 3 mL of 10 mM Tris–HCl (pH 7.5) containing 60 mM calcein and agitated for 30 min. A freeze–thaw cycle was repeated three times to obtain multilamellar vesicles (MLV). Subsequently, the suspension was passed through a polycarbonate membrane filter (pore size, 200 nm) nineteen times using a Liposofast® extruder (Avestin Inc., Ottawa, Canada) above 5° of the phase transition temperature T_m. The resultant calcein-entrapping LUV were separated from the excess amount of calcein by gel filtration using Sepharose 2B (Sigma-Aldrich, St. Louis, MO) with 10 mM Tris–HCl buffer (pH 7.5) containing 1 mM EDTA and 150 mM NaCl. The lipid and cholesterol concentration in the LUV fraction were measured using a phospholipid C-Test® (Wako Pure Chemical Industries, Ltd., Osaka, Japan) [22] and cholesterol E-test Wako® (Wako Pure Chemical Industries, Ltd., Osaka, Japan), respectively. The resulting stock solution was stored at 4 °C under nitrogen gas. For preparation of small unilamellar vesicles (SUV), the hydrated liposome suspension in the calcein-containing buffer was sonicated using Branson Sonifier 250® (Branson Ultrasonics Corporation, Danbury, CT) with a duty cycle of 50% for 30 min at temperature above 5° higher than lipid T_m. The calcein entrapped SUV liposomes were separated from excess amount of calcein described above.

Glycophorin A was reconstituted in lipid vesicles by the method of MacDonald and MacDonald [23]. Briefly, lyophilized GpA was dissolved in 1 mM Tris–HCl (pH 7.4), and added with 225 volumes of 2:1 $CHCl_3$–MeOH. To this emulsion, egg yolk lecithin dissolved in 2:1 $CHCl_3$–MeOH was added. The mixture was evaporated to form a dry lipid–protein film. Then the film was dried via a vacuum pump for at least 1 h to remove traces of solvents. The LUV liposomes were prepared from this GpA–lipid film as described earlier.

To monitor calcein leakage from LUV, 20 μL of the LUV suspension in a cuvette was diluted with the same buffer to 980 μL. A 20-μL aliquot of AM in MeOH was added to the LUV suspension, and the time course of calcein leakage from the LUV was monitored by the increase in fluorescence intensity (excitation 490 nm and emission 517 nm). Twenty micro liters of 10% Triton X-100 was added to obtain the condition corresponding to the 100% leakage.

2.4. Gel electrophoresis

The dissociation of glycophorin A (GpA) was monitored by SDS-PAGE [16]. Briefly, GpA (1.2 μg) was dissolved in 170 μL of a sample buffer containing 50 mM Tris–HCl (pH 6.8), 0.2% SDS, 30% glycerol, and 1 ppm bromophenol blue. A sample in 2 μL MeOH was added to 14 μL of the GpA solution, incubated for 1 h, and loaded onto a well for a Real Gel Plate (concentration gradient, 10–20%, Biocraft Co., LTD, Tokyo, Japan). The SDS-PAGE gel was developed for 90 min at 10–20 mA with a buffer containing 25 mM Tris, 192 mM glycine, and 0.1% SDS, and then subjected to periodate oxidation (0.2% for 15 min at 4 °C). The proteins were detected using Silver Staining Kit Protein® (Amersham Biosciences, Piscataway, NJ).

2.5. Surface plasmon resonance (SPR) and CD experiments

The interactions of amphidinols with immobilized GpA onto a SPR biosensor were monitored at 25 °C using a BIAcore X® (Biacore AB, Uppsala, Sweden). Immobilization of GpA was carried out on a CM5® sensor chip. 0.1 M of NHS and 0.4 M of EDC was injected for 7 min at a flow rate 5 μL/min. Two hundred micrograms per milliliter of GpA in 1 mM of CTAB was injected for 7 min at a flow rate 5 μL/min. Ethanol amine was then injected for 7 min at a flow rate 5 μL/min. AMs (1–100 μM) in the running buffer (HBS-EP) containing 10 mM HEPES (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, and 0.005 % surfactant P20 were injected to the GpA-immobilized sensor chip for 120 s at 20 μL/min. The buffer was replaced with the standard running buffer, and the dissociation of bound AM was monitored for 60 s at a 20 μL/min. For regeneration of the sensor surface, it was subjected to a continuous flow of the standard running buffer for 4 min cycle.

GpA (2.6 μM) was mixed with or without AM in 0.1% SDS at room temperature for 1 h. Delta ε was determined on a JASCO® spectropolarimeter (J-720 WO, Jasco Corporation, Tokyo, Japan) in a quartz cuvette (path length, 0.2 mm; band width, 1.0 nm). Ten repetitive scans in the wavelength from 198 nm to 250 nm were measured at 25 °C to be integrated.

3. Results

3.1. Comparison between erythrocytes and liposomes in terms of AM activity

The hemolytic assays and liposome permeabilizing experiments of AMs were carried out under similar conditions, particularly for phospholipid concentrations. As shown in Fig. 2, AM2, AM3, AM6 and AM7 showed higher efficacies to erythrocytes than those to liposomes of egg yolk lecithin, which mainly consisted of PC, PE, triglyceride and cholesterol, and mimicked the lipid composition of erythrocyte membrane; in a previous study[13], we evaluated the activity of AM using liposomes comprised of 90% POPC (>99%) and 10% cholesterol, EC_{50} values of AM2 and AM3 are 3.0 and 2.5 μM, respectively, which are comparable with the values in egg yolk PC (5.0 and 4.4 μM depicted in Fig. 2). These results imply that minor constituents in the lecithin did not significantly influence the activity of AMs. AM3 is eleven times more efficacious in hemolysis than in membrane permeabilization of liposomes. The enhanced potencies in hemolysis are less prominent for the other AMs; three times with AM2, four times with AM6 and five times with AM7 (Fig. 1, Table 1).

3.2. Glycophorin A as a model protein for AM's binding to erythrocytes

The results in Fig. 2 suggest the presence of binding proteins in erythrocyte membranes. To further examine the interaction between AM and proteins, we became interested in GpA since AMs are assumed to interact with membrane integral proteins

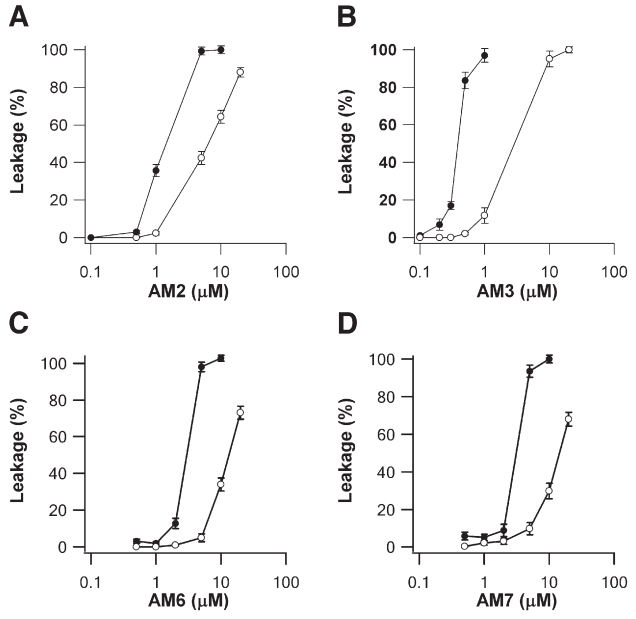


Fig. 2. Dose-dependent activity of AMs to the red blood cells and LUV stimulated by AM2 (A), AM3 (B), AM6 (C) and AM7 (D). Closed circles (●) and open circle (○) denote hemolysis and liposome permeabilization activities, respectively. Activity to red blood cells was measured by OD_{450}, and leakage of calcein from LUV was monitored by change in fluorescence (excitation: 470 nm; emission, 520 nm). In all cases, the lipid concentration was 27 mM.

Table 1
Hemolytic and antifungal activities of amphidinols

Compound	AM2	AM3	AM6	AM7	AM14	AM15	dsAM7
Antifungal[a] (MEC in μg/disk; *Aspergillus niger*)	6	6	6	10	>60	60	8
Hemolysis[b] (EC_{50} in μM; human erythrocytes)	1.7	0.4	2.9	3	>50	>50	1.2

[a] The maximum amount tested was 60 μg/disk.
[b] The maximum concentration tested was 50 μM.

of erythrocyte among which GpA with a single transmembrane α-helix should provide a simple structural model. AM2 was incubated with GpA in an aqueous phase and then added to erythrocytes (Fig. 3A). The binding of AM2 to the GpA in media inhibited, and eventually abolished hemolytic activity; Fig. 3A showed the inhibition of hemolysis with increasing concentrations of GpA. The high concentration of GpA was, however, required for completely abolishing hemolysis [24]. Fig. 3B depicted the inhibitory effect as viewed in the dose–response curve of AM2 after incubation with GpA monoclonal antibody, indicating that interaction between GpA and its antibody markedly reduced the efficacy of AM2. The addition of other soluble proteins such as bovine serum albumin did not affect hemolytic activity of AM.

3.3. Examination of the interaction between AM and GpA by SDS-PAGE

GpA has a single transmembrane helix [24], where GpA undergoes dimerization [21,25] by interaction mainly between the GXXXG residues in α-helices called “glycine zipper” [21]. In addition, GpA tends to aggregate under aqueous conditions even in the presence of detergents [24]. It is reported that GpA migrated on SDS-PAGE as dimers or oligomers, which can be dissociated into monomers by peptides corresponding to the transmembrane domain named GpA-TM [24]. The dissociation can, thus, be accounted for by direct binding of GpA-TM to the corresponding domain of GpA [26]. We previously used this

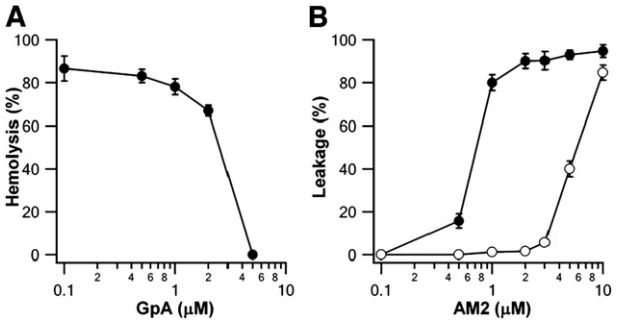


Fig. 3. Inhibition of AM-induced hemolysis by preincubation with GpA (A) and with GpA antibodies (B). (A) AM2 at 4 μM (●) was preincubated with various concentrations of GpA, and then added to an erythrocyte suspension. Hemolytic activities induced by AMs were plotted versus GpA concentration. (B) Erythrocytes were preincubated with (○) and without (●) anti GpA monoclonal antibody, and then treated with AM2. Hemolytic activities induced by AMs were plotted versus AM2 concentration.

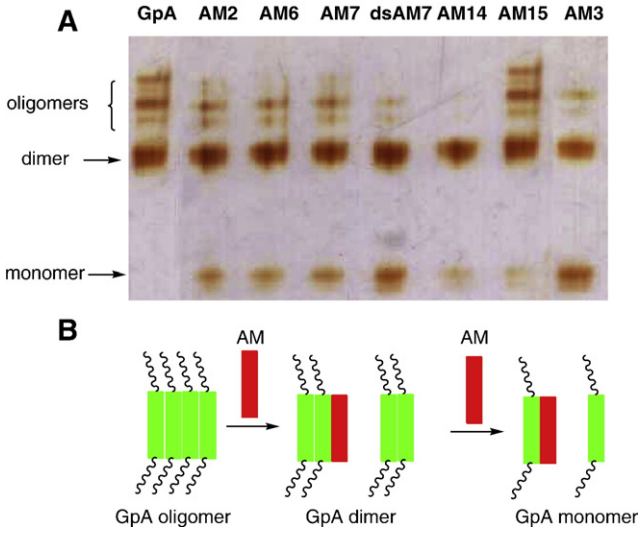


Fig. 4. Interaction between AMs and GpA as revealed by SDS-PAGE. (A) SDS-PAGE gel after silver staining. In aqueous 0.1 % SDS, 2.6 pmol of GpA was preincubated with 10 nmol of AM, then loaded onto a 10–20% acryl amide gradient gel, and run for 2 h at 10 mA: from the left, control GpA alone, AM2, AM6, AM7, dsAM7, AM14, AM15, and AM3. (B) A hypothetical model for GpA–AM interaction to induce dissociation of GpA oligomers to dimers and then to monomers.

method to evaluate the interaction of a polyether compound, yessotoxin, with GpA [27,28]. With the same method we examined the affinity between AM and GpA. In Fig. 4, the monomer bands predominantly appeared at the lanes of AM3 and dsAM7, whereas showed only faint bands at the lanes of AM14 and AM15 both of which possess a dihydroxyl group at the polyene terminus. These results are parallel with their biological activity (Table 1). On the other hand, dissociation of oligomers to dimers is apparent for AM14, suggesting that a different mechanism is involved in this process.

Table 2
K_d values for the interaction between GpA-TM and amphidinols estimated by surface plasmon resonance

Amphidinol	K_d (μM)	
	GpA	GpA-TM
AM2	65.0	22.9
AM3	80.7	15.1
AM6	48.0	17.0

3.4. Detection of the interaction between AM and GpA by surface plasmon resonance

In the next step, we examined the affinity of AMs to GpA in a semiquantitative manner using a surface plasmon resonance (SPR) method. Dissociation constants between AM and the proteins immobilized on a chip can be estimated by SPR. As shown in Fig. 5, binding of AMs to GpA was concentration-dependent, which revealed that the K_d values of their interactions were of the order of 10 μM. To determine the site of the interaction in GpA, the transmembrane domain of GpA (GpA-TM) was immobilized, which consisted of 29 amino acid residues [21], EPEITLIIFGVMAGVIGTILLISYGIRRL. SPR experiments showed K_d values of the same order of magnitude as those for GpA (Table 2), which clearly indicated the transmembrane domain to be a target of interaction for AMs with GpA.

Another example of transmembrane proteins, we examined the affinity between APH-1 and AM3 by SPR experiments; APH-1 is a multipass integral protein rich in α-helix structure. APH-1 showed a similar affinity to GpA with the K_d value of 26 μM. On the other hand, AM3 had no detectable interaction with soluble proteins such as bovine serum albumin and avidin (data not shown).

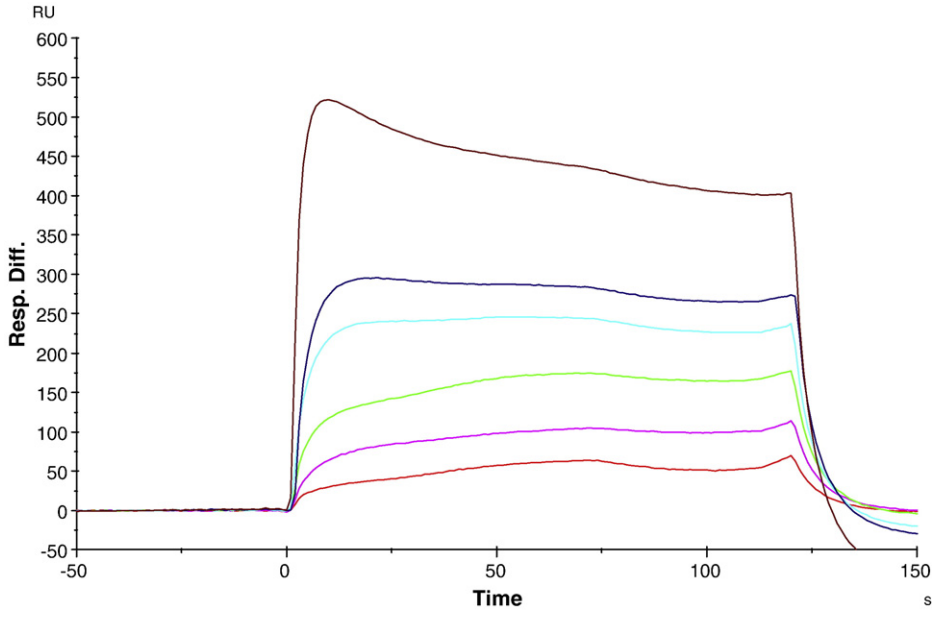


Fig. 5. SPR sensorgrams for recording the interaction of AM3 with GpA immobilized on the surface of CM5® sensor chip. AM3 concentrations introduced to the sensor chip were 100, 50, 30, 20, 10 and 5 μM, which corresponded to the sensorgrams from the top to the bottom.

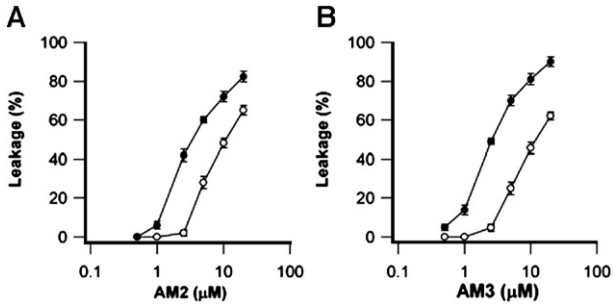


Fig. 6. AM-induced calcein leakage from GpA-reconstituted liposomes. Dose–response curves of calcein leakage activity for (A) AM2 and (B) AM3 to liposomes with (●) or without (○) reconstituted GpA are shown. The lipid concentration was 27 mM.

3.5. Effect of reconstituted GpA in liposomes on AM membrane-permeabilizing activities

The results hitherto obtained suggest that AMs interact with a membrane integral protein, which may account for the higher efficacy of AMs in hemolysis than in liposome permeabilization. To confirm this notion, the membrane-permeabilizing activity of AMs was investigated with GpA-reconstituted liposomes, which were composed of egg yolk lecithin containing approximately 1% cholesterol. Fig. 6 demonstrates that AM activity is significantly potentiated in the presence of GpA. As compared in EC_{50}, AM3 and AM2 are around five times more sensitive to liposomes reconstituted with GpA than those without GpA, clearly indicating that membrane proteins on erythrocyte membrane such as GpA play a role in potentiating the AM activity.

4. Discussion

To our knowledge, this is the first report to describe the presence of proteins that potentiate the membrane-permeabilizing activity of AMs. In previous studies, we have shown that AMs exhibit the membrane activities to protein-free liposomes [11,13]. In our initial experiments (Fig. 2), it was shown that membrane constituent(s) in erythrocyte membranes enhance the AMs-induced hemolytic activity. Since erythrocyte membranes are rich in oligosaccharide chains attached to the membrane-embedded proteins and lipids [29], we examined the effect of neuraminidase on the hemolytic activity induced by AMs. The enzyme hydrolyzes virtually all the protein/lipid-bound neuraminic acid, the major terminal sugar of GpA glycosyl chains [17,30]. However, the enzyme treatment did not significantly modify the efficacy of AMs (data not shown), ruling out the possibility that the sialic acid moieties play a major roll in the potentiation of the AM activity.

In the present study, we obtained lines of experimental evidence supporting the interaction between AM and GpA; (a) preincubation of AM with GpA reduced the hemolytic activity (Fig. 3A); (b) SDS-PAGE experiments showed that AMs dissociated GpA oligomers and dimers into monomers (Fig. 4); (c) SPR experiments using an immobilized GpA chip demonstrated that AM3 binds to GpA with moderate affinity (Fig. 5); and (d) incorporation of GpA into liposomes consisting of egg yolk lecithin enhanced the AM activity (Fig. 6). In addition, the interaction between AMs and GpA was shown to occur with the GpA-transmembrane domain since the corresponding peptide, GpA-TM, has approximately similar (or even higher) K_d values as that of the whole GpA proteins in the SPR experiments. The inhibitory effect by GpA antibodies (Fig. 3B) may be another evidence for their interaction. The antibodies largely bind to the extracellular chains of GpA, which may imply that the hydrophilic chain comprising polysaccharides and peptides may also play a role in potentiating AM activity. The higher affinity to GpA-TM than GpA (Table 2) and insignificant effects of neuraminidase, however, reduce the possibility that the extracellular chains make a major contribution to this potentiation.

In the SPR experiments, GpA covalently bound on a sensor chip is partly covered by surfactant. As Bormann et al. reported [16] that GpA takes an α-helix structure for its transmembrane parts in SDS, GpA and GpA-TM are assumed to take an α-helix structure under the SPR conditions. Thus, K_d values shown in Table 2 may be those between α-helix peptides and AMs. As compared with the EC_{50} concentrations of hemolysis in Table 1, these K_d values are much larger, which indicates that AMs scarcely bind to GpA on the erythrocyte membranes in efficacious concentrations. The same should be the case with the reconstituted liposomes (Fig. 6). How can GpA enhance the activity of AMs without directly binding to AMs? One of the plausible answers is that membrane integral peptides such as GpA-TM increase the affinity of AMs to membrane, in other words, the partition coefficient of AMs to membrane is greater for protein-containing bilayers than that for pure lipid ones. Since AMs form pores/lesion in phospholipid layers in a sterol-dependent manner at μM concentrations [11,13], biological activities including the antifungal are largely due to this pore formation. Yet, the great variations among AM homologues in membrane affinity [8] and the difference of AM activity in the presence or absence of reconstituted GpA (Fig. 6) may suggest that some unknown membrane proteins with higher affinity to AMs are responsible for their potent activities against fungi and erythrocytes. The effect of GpA alone is not enough to explain the significant difference in efficacy between erythrocytes and GpA-reconstituted liposomes. This inconsistency can be explained by the presence of other constituents in erythrocyte membranes, which further enhance the activity probably by increasing an amount of AMs bound in membranes. Detailed investigations of the interactions between antifungal agents and membrane proteins may provide invaluable information as to how to improve the pharmacological properties and how to suppress the side effects.

5. Conclusion

The present study clearly demonstrated that the membrane-permeabilizing activity of AMs is significantly enhanced by the presence of a membrane integral peptide, GpA. The interactions between AMs and GpA or its transmembrane peptide, GpA-TM, were evidenced by SDS-PAGE, surface plasmon resonance, and GpA-reconstituted liposome experiments. Upon comparing the efficacy in membrane disruption and the K_d values estimated from SPR, it is suggested that the potentiation of AM activity by

GpA is due to the higher affinity of AMs to protein-containing bilayers.

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