Comparative study of the membrane-permeabilizing activities of mastoparans and related histamine-releasing agents in bacteria, erythrocytes, and mast cells

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ABSTRACT

The membrane-permeabilizing activities of mastoparans and related histamine-releasing agents were compared through measurements of K^+ efflux from bacteria, erythrocytes, and mast cells. Changes in bacterial cell viability, hemolysis, and histamine release, as well as in the shape of erythrocytes were also investigated. The compounds tested were mastoparans (HR1, a mastoparan from *Polistes jadwagae*, and a mastoparan from *Vespula lewisii*), granuliberin R, mast cell-degranulating peptide, and compound 48/80, as well as antimicrobial peptides, such as magainin I, magainin II, gramicidin S, and melittin. We used a K^+-selective electrode to determine changes in the permeability to K^+ of the cytoplasmic membranes of cells. Consistent with the surface of mast cells becoming negatively charged during histamine release, due to the translocation of phosphatidylserine to the outer leaflet of the cytoplasmic membrane, histamine-releasing agents induced K^+ efflux from mast cells, dependent on their ability to increase the permeability of bacterial cytoplasmic membranes rich in negatively charged phospholipids. The present results demonstrated that amphiphilic peptides, possessing both histamine-releasing and antimicrobial capabilities, induced the permeabilization of the cytoplasmic membranes of not only bacteria but mast cells. Mastoparans increased the permeability of membranes in human erythrocytes at higher concentrations, and changed the normal discoid shape to a crenated form. The structural requirement for making the crenated form was determined using compound 48/80 and its constituents (monomer, dimer, and trimer), changing systematically the number of cationic charges of the molecules.

Keywords: Mastoparan; Compound 48/80; Amphiphilic peptide; Antimicrobial peptide; Histamine-releasing agent; Membrane permeability; Cell shape

1. Introduction

Amphiphilic peptides can interact with membrane phospholipids, independently of specific receptors, in a rapid and concentration-dependent way [1−9]. These peptides generally form a cationic amphiphilic secondary structure, with hydrophobic and cationic amino acid residues in opposite directions, and therefore, can interact with membrane lipids through both electrostatic and hydrophobic interactions. The bacterial cytoplasmic membrane presents an outermost leaflet rich in negatively charged phospholipids, while the external face of mammalian cells is predominantly composed of zwitterionic phospholipids. Besides the zwitterionic lipids, the mammalian cells have high concentrations of cholesterol, which acts to stabilize the bilayer. The lipid composition of the cell membrane has been shown to be of paramount importance to the selectivity of the permeabilization activity. An important feature of amphiphilic peptides showing antimicrobial activity is positively charged residues distributed along the peptide chain, serving to generate an amphiphilic structure. The presence of these charged groups generally renders the peptide more active against bacteria. Moreover, due to weak interaction with zwitterionic lipids and cholesterol, most of the peptides act weakly on mammalian cells.

In the present study, we were particularly interested in the influences of mastoparans and related histamine-releasing reagents on the permeability of the cytoplasmic membrane of mast cells, compared with the membranes of bacterial cells and erythrocytes. Mastoparans and many cationic substances activate rat peritoneal mast cells, leading to the rapid release of histamine by exocytosis [10−12]. These agents trigger mast cell exocytosis by directly activating G proteins that control exocytosis [13−16], and hence, the secretion of histamine generally proceeds without changes to the permeability of the plasma membrane of mast cells.

In secretory cells, however, the transbilayer movement of an anionic phospholipid from the inner to outer leaflet of the plasma membrane is known to occur during platelet activation and exocytotic release from mast cells [17−19]. Thus, it was speculated that mastoparans, which act weakly on mammalian cells but strongly on bacterial cells, may enhance permeabilization in mast cells, because the surface of mast cells becomes negatively charged during histamine release, due to the translocation of phosphatidylserine to the outer leaflet of the cytoplasmic membrane [19].

To clarify the actions of amphiphilic peptides in terms of cell membrane dependence in detail, we compared the membrane-permeabilizing activities of various mast cell-degranulating peptides and antimicrobial peptides using three different types of cells; rat peritoneal mast cells, human erythrocytes, and *Staphylococcus aureus* cells. The compounds tested included mastoparans (such as HR1 from the giant hornet *Vespa orientalis* [20], mastoparan from *Polistes jadwagae* [11], and mastoparan from *Vespula lewisii* [10,11,21]), granuliberin R [22], mast cell-degranulating peptide [23], and a synthetic histamine liberator, compound 48/80 [24,25], as well as antimicrobial peptides, such as magainin I [26], magainin II [26], gramicidin S [27−29], and melittin [28,29]. The sequences of peptides and the structure of compound 48/80 and its constituents are shown in Table 1 and Fig. 1, respectively. They all possess a cationic amphiphilic structure. We used a K^+-selective electrode to monitor changes in the permeability of the cytoplasmic membrane of cells. Because the internal ionic environment of bacterial and mammalian cells is generally rich in K^+, leakage of this ion has been used to monitor membranolytic events in various cell systems [21,27,29,30]. We present here for the first time evidence that amphiphilic peptides, possessing both histamine-releasing and antimicrobial capabilities, enhanced the permeability to K^+ of not only bacteria but mast cells.

Although mastoparans, like many amphiphilic peptides tested in this study, acted weakly on erythrocyte membranes, we were particularly interested in the morphological changes of human erythrocytes induced by the peptides, having previously observed that amphiphilic peptides, melittin and gramicidin S, caused changes in the normal discoid shape of human erythrocytes to produce a crenated form at the concentrations causing the change in permeability [29]. Such a direct observation of morphological change seemed to provide significant information on the mechanism of action of amphiphilic compounds. The barrel-stave and carpet models have been proposed to explain the permeabilization of membranes by amphiphilic peptides [1−8]. In the barrel-stave model, peptide monomers associate and form a bundle of helices embedded in the membrane, forming a transmembrane channel. In the carpet-like model, the peptides cover the membrane like a carpet and remain there with their hydrophobic surface facing the membrane and their hydrophilic surface facing the solvent. According to this model, the bilayer is destroyed by the formation of toroidal pores, micelles, and vesicles. After the treatment of human erythrocytes with mastoparans, changes from a normal discoid to crenated form were observed, showing the accumulation of the peptides at the outer surface of the membrane. This finding supported that mastoparans increased the permeability of the erythrocyte membrane as proposed by the carpet model [4,31]. The structural requirement for making the crenated form was further investigated with the use of compound 48/80 and its constituents (monomer, dimer, and trimer) [32], changing systematically the number of cationic charges of the molecules. Compound 48/80 is a polycationic polymer prepared by condensation of *p*-methoxy-*N*-methylphenethylamine and formaldehyde, and the most active polymer is the hexamer [24,25], as shown in Fig. 1.

2. Materials and methods

2.1. Reagents

The sources of reagents were as follows: mastoparan HR1 (named mast cell-degranulating peptide HR1 in the Sigma catalog), mastoparan from *Polistes jadwagae* (hereafter, *Polistes* mastoparan), mast cell-degranulating peptide (MCD peptide), granuliberin R, gramicidin S, melittin, magainin I, magainin II, compound 48/80, and valinomycin from Sigma (St. Louis, MO); mastoparan from *Vespula lewisii* (*Vespula* mastoparan) from Peptide Institute (Osaka, Japan); dioctyl phthalate from Tokyo Kasei (Tokyo, Japan); and poly(vinyl chloride) (PVC) (degree of polymerization, 1020) from Nacalai Tesque (Kyoto, Japan). The monomer, dimer, and trimer of compound 48/80 were prepared as reported previously [32]. All other materials were of analytical reagent grade.

2.2. Electrode system

The K^+-selective electrode was constructed using a PVC-based membrane as reported previously [30,33]. The PVC membrane had the following composition: 1 mg of valinomycin, 60 μl (about 60 mg) of dioctyl phthalate, and 30 mg of PVC. The materials were dissolved in tetrahydrofuran (about 1 ml) and poured into a flat Petri dish (28 mm in diameter). Then, the solvent was evaporated off at room temperature. The resulting membrane was excised and attached to a PVC tube (4 mm in outer diameter and 3 mm in inner diameter) with tetrahydrofuran adhesive. The PVC tube was filled with 10 mM KCl, and the sensor membrane was conditioned overnight. The electrochemical arrangement was Ag,AgCl/10 mM KCl/sensor membrane/sample solution/1 M NH_4NO_3 (salt bridge)/10 mM KCl/Ag,AgCl. The electromotive force (emf) between the silver/silver chloride electrodes

was measured using a voltmeter with high input impedance produced by a field-effect transistor operational amplifier (LF356, National Semiconductor, Sunnyvale, CA; input resistance $>10^{12}$ Ω) and was recorded.

2.3. Bacteria and erythrocytes

S. aureus FDA 209P cells were grown at 37°C in a medium containing 1.5% polypeptone, 0.5% bovine extract, 0.5% NaCl, and 0.5% K_2HPO_4 [27]. The cells were harvested in an exponential phase of growth, washed twice with buffer (100 mM choline chloride and 50 mM 4-morpholinepropanesulphonic acid (Mops)−Tris, pH 7.2), and suspended in this buffer at 5×10^9 cells/ml (0.6 mg cell protein/ml). Protein content was determined by the method of Lowry et al. [34]. Human erythrocytes were suspended in 0.15 M NaCl and 5 mM 4-(2-hydroxyethyl)-1-peperazineethanesulphonic acid (Hepes)−NaOH (pH 7.4) at 1% hematocrit of 1×10^8 cells/ml. Cells were incubated with reagent at 37°C for 30 min, and the amount of K^+ effluxed was measured with a K^+-selective electrode [29]. The total amount of K^+ was determined by disrupting cells with melittin [29]. The viability of cells was determined by counting colonies, and hemolysis was estimated by measuring the absorbance at 540 nm [29].

2.4. Mast cells

Mast cells were collected from the peritoneal cavities of male Wistar rats (300−350 g) and purified using Percoll as reported previously [35]. The mast cells were suspended in a K^+-free buffer comprising 0.154 M NaCl, 0.9 mM $CaCl_2$, 5.6 mM glucose, 0.01% bovine serum albumin, and 5 mM Hepes−NaOH (pH 7.4) at 8×10^5 cells/ml. Because a great deal of spontaneous efflux of K^+ was observed at 37°C, this experiment was carried out at a lower

temperature, 20°C. We monitored the time course of the efflux from mast cells in situ to avoid any experimental error arising from a spontaneous efflux, minimizing the difference in electric potential of the K^+-selective electrode before and after addition of the reagent. Furthermore, we measured the efflux of K^+ from mast cells by increasing the cell concentration to 8×10^5 cells/ml, using a miniaturized measuring system. This made the results more reliable, compared with a previous study [21]. Specifically, the K^+ and histamine assays proceeded as follows. The K^+ and reference electrodes were immersed in a mast cell suspension (200 μl), and the suspension was constantly stirred with a stirrer bar. The electrode system was compact [30], and therefore, as little as 200 μl could be measured. After the change in potential resulting from K^+ efflux was recorded for 5 min, 50 μl of the cell suspension was pipetted to analyze histamine release, and melittin (final concentration, 50 μM) was added to the remaining cell suspension to determine the total amount of K^+ in mast cells. The amount of histamine was determined fluorometrically after conversion to the fluorescent product by a reaction with *o*-phthalaldehyde as reported [36].

2.5. Morphological observations

Cells were fixed with 2% glutaraldehyde dissolved in 1/30 M NaH_2PO_4/Na_2HPO_4 (pH 7.4) and observed under an optical microscope connected to a monitor [37,38]. Shape changes were quantified using the morphological indices defined by Fujii and co-workers [39].

3. Results

3.1. K^+ permeability measurements using mast cells

Permeability measurements using a K^+-selective electrode are widely performed using

bacteria and erythrocytes, as well as artificial liposomes [27,29,30,33,40−43]. In this study, we applied the electrode to mast cells. Because the difference in the electric potential of the K^+-selective electrode before and after the addition of reagents was relatively small, we monitored the time course of the efflux of K^+ from mast cells in situ. Fig. 2a shows the calibration graph for the K^+ electrode in a K^+-free buffer comprising 0.154 M NaCl, 0.9 mM $CaCl_2$, 5.6 mM glucose, 0.01% bovine serum albumin, and 5 mM Hepes−NaOH (pH 7.4). Fig. 2b shows the effect of HR1 on the efflux from mast cells. Addition of the cell suspension produced an increase in the K^+ concentration, which was attributed to the K^+ remaining in the cell suspension. Addition of HR1 (10 μM) induced an increase in the K^+ concentration with a short time lag. Finally, a high concentration of melittin (50 μM) was added to disrupt the membrane of mast cells, in order to determine the total amount of K^+ contained in the cells. Thus, it was calculated that HR1 (10 μM) induced 48% efflux within 5 min from the calibration graph shown in Fig. 2a. Such direct monitoring presented unambiguous evidence of the efflux of K^+ from mast cells. Furthermore, the fact that the efflux of K^+ began to increase shortly after addition of HR1 correlated with the time-required release of histamine [44] accompanying the translocation of phosphatidylserine to the outer leaflet of the cytoplasmic membrane.

3.2. K^+ efflux, cell viability, hemolysis, and histamine release

Fig. 3 shows HR1-induced K^+ efflux, cell viability, hemolysis, and histamine release in *S. aureus* cells, erythrocytes, and mast cells. As shown in Fig. 3a, this peptide lowered the viability of *S. aureus* cells at the concentrations causing changes in permeability to K^+, indicating that the antimicrobial activity of HR1 was the result of increased membrane permeability, as in the case of many antimicrobial peptides [1−9]. This peptide also caused

the efflux of K^+ from erythrocytes and hemolysis at higher concentrations. The weak activity against mammalian cells is a general characteristic of amphiphilic peptides [1−9]. An interesting feature is that the concentrations required for the efflux from mast cells were significantly lower than those used with erythrocytes. This is most probably because the surface of mast cells became negatively charged during the secretion of histamine, due to the translocation of phosphatidylserine to the outer leaflet of the cytoplasmic membrane of mast cells [19], making the peptide susceptible to mast cells, as mentioned in the Introduction. Furthermore, HR1 induced K^+ efflux from mast cells immediately after liberating histamine. This indicated that HR1 had the ability to interact with negatively charged lipids on mast cells, in accordance with the strong effect on bacterial membranes rich in anionic lipids, as shown in Fig. 3a. Similar results were obtained with *Polistes* mastoparan and *Vespula* mastoparan. Table 2 summarizes ED_{50} values (concentrations giving 50% K^+ efflux, cell viability, hemolysis, or histamine release) for these mastoparan groups, along with the results for related histamine-releasing agents mentioned later.

Next, we examined the effects of granuliberin R (Fig. 4). This peptide, isolated from frog skin, has been reported to liberate histamine from rat peritoneal mast cells [22], though no study has reported antimicrobial activity. In fact, granuliberin acted very weakly on *S. aureus* cells, while it induced the release of histamine from mast cells near the concentration range of HR1. No remarkable K^+ efflux from erythrocytes or hemolysis was observed. Interestingly, the efflux from mast cells occurred close to the concentration range causing K^+ efflux from *S. aureus* cells, though at very high concentrations. This finding seemed to support the view that the peptide acted on anionic lipids of not only bacterial cells but mast cells that appeared during histamine secretion, though the concentrations needed to permeabilize membranes were very high. MCD peptide [23], a potent histamine liberator

found in bee venom, did not remarkably induce K^+ efflux from mast cells, consistent with the absence of antimicrobial activity at the concentrations tested. The ED_{50} values (the concentrations giving 50% K^+ efflux, cell viability, hemolysis, and histamine release) for granuliberin R and MCD peptide are summarized in Table 2.

Fig. 5 shows the results for compound 48/80. This compound is known to effectively liberate histamine via a selective and noncytotoxic action [45], and also to act as a weak antimicrobial agent [46,47]. Consistent with this, it effectively liberated histamine from mast cells at low concentrations, and reduced the viability of *S. aureus* cells at relatively high concentrations. Compound 48/80 decreased the viability of *S. aureus* cells concomitantly with the efflux of K^+, showing that the antimicrobial activity was attributable to the increased permeability of the bacterial cytoplasmic membrane. The efflux from mast cells was induced at a much lower concentration range than that from erythrocytes, and rather near to the concentrations required for K^+ efflux from *S. aureus* cells, also indicating the susceptibility of the histamine-releasing agent to negatively charged membranes.

The same experiments were performed using various membrane-active peptides; magainin I, magainin II, gramicidin S, and melittin. Table 2 summarizes ED_{50} values for all substances tested in this study. In general, there was a tendency for the amphiphilic peptides, with both histamine-releasing and antimicrobial capabilities, to induce the permeabilization of the cytoplasmic membranes of not only bacteria but mast cells.

3.3. Morphological changes in human erythrocytes

Fig. 6 shows mastoparan-induced morphological changes in human erythrocytes at the concentrations causing hemolysis (about 20−30%). All the mastoparans caused a change from the normal discoid shape to a crenated form, as in the case of gramicidin S and melittin

reported previously [29]. The crenated cells increased with increasing concentrations of the peptides.

Changes in the shape of human erythrocytes induced by amphiphilic compounds are generally discussed based on the bilayer couple hypothesis proposed by Sheetz and Singer [48]. According to this hypothesis, cell shape is strongly dependent on the charge of the amphiphiles. Negatively charged amphiphiles caused discoid cells to adopt a crenated form named echinocytes, while positively charged amphiphiles with proper lipophilicity generated an invaginated form named stomatocytes. Anionic amphiphiles that produce echinocytes associated mostly with the outer monolayer of the erythrocyte membrane, as a result of their inability to cross the bilayer, or because of repulsion by a negatively charged phosphatidylserine located in the inner monolayer of the erythrocyte membrane [49]. Conversely, cationic amphiphiles that induced stomatocytes did so by partitioning selectively into the cell inner monolayer, through association with an inner anionic lipid. However, the cationic compounds, whose rates of transmembrane movement were slow, made the erythrocytes crenated [39,50]. The mastoparans tested in this study changed the shape to that of echinocytes. These peptides had several basic amino acids, such as lysine, and also acidic amino acid, such as aspartic acid, in *Polistes* mastoparan, making it strongly hydrophilic, and hence, they had difficultly crossing the lipid interior, causing selective accumulation in the outer monolayer, leading to the formation of echinocytes. To obtain further insight into the dependence of changes in cell shape on the number of cationic charges, we used compound 48/80 and its components (monomer, dimer, and trimer). Dialysis and gel filtration [24] and NMR studies [25] have shown that the most active polymer is probably the hexamer. Fig. 7 shows the morphological change of human erythrocytes induced by compound 48/80 and its components, at the same concentration (0.2 mg/ml). The monomer

did not induce marked changes in morphology at this concentration, probably due to low partition in membranes. The dimer caused a change to the invaginated form, indicating that it was preferentially incorporated into the inner monolayer of the membrane by interacting with an inner anionic phospholipid, because it was more lipophilic than the monomer. However, the trimer suppressed the change to the invaginated form, and moreover, 48/80 induced echinocytes to form. These results indicated that the increased number of cationic charges producing a strong hydrophilic nature made it difficult for the compounds to traverse the lipid interior. We expressed the dose-dependence of the change in shape using morphological indices defined by Fujii and co-workers [39]. As shown in Fig. 8, the dimer induced changes to the higher stages of the invaginated form with increases in the concentration, while the trimer suppressed a change in the invaginated form, and 48/80 induced morphological change in the opposite direction, making echinocytes. Compound 48/80 induced hemolysis (about 20%) at a concentration of 0.2 mg/ml. This feature was similar to mastoparans, inducing hemolysis via echinocytes.

4. Discussion

In this study, we made potentiometric measurements with a K^+-selective electrode to analyze the actions of cationic amphiphilic compounds on *S. aureus* cells, erythrocytes, and mast cells. The use of an ion-selective electrode for biochemical analysis is attractive, because the measurement is fast, easy, and continuous in a turbid cell suspension. Hence, ion-selective electrodes have widely been applied to the assay of membrane permeability [27,29,30,33,40−43]. For analyzing changes in the permeability of cytoplasmic membranes of living cells, the use of a K^+-selective electrode is quite effective, because K^+ is present in

the cytoplasm of all kinds of cells, and the permeability-increasing action of biologically active substances in different cells can be easily compared. A drawback is the relatively low sensitivity of the electrodes which requires a high concentration of cells. With bacteria and erythrocytes, it is easy to prepare a large number of cells; however, for mast cells, preparing large numbers is rather difficult. We solved this problem by using small volumes of cell suspension (200 μl) to enhance the concentration of cells. This made it possible to perform 5−6 measurements from 10^6 mast cells, which could usually be collected from the abdominal cavity of an individual rat.

Mastoparans generally exhibit low hemolytic activity, but relatively high antimicrobial activity [21]. All the mastoparans tested in this study (HR1, *Polistes* mastoparan, and *Vespula* mastoparan) gave just such results. Several characteristics of the peptides' actions should be mentioned. First, all mastoparans increased the permeability of *S. aureus* cells to K^+, and decreased cell viability at similar concentrations, supporting that the antimicrobial activity of mastoparans was attributable to the increased permeability of the bacterial membrane. Second, mastoparans were more effective in increasing the permeability of mast cells than erythrocytes. Because the release of histamine eventually produced a negative charge in mast cells, the efflux of K^+ from mast cells was observed after histamine's secretion. Third, the finding that the concentrations required for the efflux of K^+ from *S. aureus* cells were much lower than those needed for the release of histamine indicates that the mastoparans acted as antimicrobial agents rather than histamine liberators. Antimicrobial peptides, magainin I and magainin II, acted on bacteria and mast cells, similarly to the mastoparans, though these peptides were less effective against erythrocyte membranes than mastoparans. Peptides acting strongly against both bacteria and erythrocytes, such as melittin and gramicidin S, functioned at similar concentrations. An interesting feature is that

mastoparans and magainins, whose antimicrobial activity was stronger than the histamine-releasing activity, increased K^+ efflux from mast cells, near to the concentration causing histamine release. This indicated that the membranolytic action of these peptides on mast cells was strong, because of the high affinity for anionic lipids. Indeed, *Vespula* mastoparan has been reported to show a cytotoxic effect, due to the permeabilization of the plasma membrane of mast cells [51], similarly to melittin [52]. Granuliberin R and MCD peptide acted much more selectively as histamine liberators than the mastoparans and magainins. These peptides could not interact with anionic lipids sufficiently to permeabilize the membrane, probably because the separation between the cationic and hydrophobic amino acid residues was insufficient to generate an effective amphiphilic structure in the molecules [53]. At high concentrations, however, granuliberin R induced K^+ efflux from mast cells and bacterial cells, supporting the view that the peptide acted on anionic lipids of not only bacterial cells but mast cells during histamine secretion. Interestingly, compound 48/80, a potentially active histamine liberator, is also a weak antimicrobial agent [46,47]. In fact, compound 48/80 induced histamine release at very low concentrations, while relatively high concentrations were required to induce the efflux of K^+ from bacteria. The concentration required for the efflux from mast cells was significantly lower than that needed for the efflux from bacteria. This may be attributable to a difference in susceptibility to compound 48/80 among anionic membranes in different environments, because the effects of amphiphilic compounds including 48/80 are known to differ among bacteria, despite the bacterial cytoplasmic membranes being generally rich in negatively charged lipids [5,26,47]. Summarizing the present results, we showed for the first time that histamine-releasing agents, also possessing antimicrobial activity, caused the permeabilization of membranes in both bacteria and mast cells.

We further investigated the morphological changes to human erythrocytes induced by mastoparans. All the mastoparans caused a change from the normal discoid shape to a crenated form (echinocytes) at the concentrations causing hemolysis. In general, cationic amphiphilic compounds cause invaginated forms named stomatocytes, as a result of electric interaction with phosphatidylserine that is concentrated in the inner leaflet of the cytoplasmic membrane [49]. However, the hydrophilicity of cationic compounds is also known to induce the formation of echinocytes, because of the reduced permeability of the agents through the membrane interior [39,50]. There are several basic amino acids, such as lysine, and also acidic amino acid, such as aspartic acid, in *Polistes* mastoparan, making it strongly hydrophilic, and hence, the peptides predominantly accumulated in the outer monolayer of the erythrocyte membrane, forming echinocytes. To obtain further insight into the dependence of cationic charges on cell shape, we used compound 48/80 and its components (monomer, dimer, and trimer). The most active constituent of compound 48/80 is reported to be the hexamer. Thus, the cationic charges of the monomer, dimer, trimer, and compound 48/80 increased to +1, +2, +3, and +6, respectively. The monomer did not induce marked changes in morphology, because of the low activity to permeate the membrane. It is well established that appropriate hydrophobicity is required to induce changes of shape in erythrocytes [54,55]. The dimer caused a change to an invaginated form. Because the dimer was more lipophilic than the monomer, it could easily permeate the membrane, further traverse the lipid interior, and reach the inside of the membrane by interacting with anionic phospholipids that are concentrated in the inner half of the membrane. The trimer suppressed the change to the invaginated form, and 48/80 generated echinocytes. These results indicate that an increase in cationic charge, enhancing hydrophilicity, made it difficult to cross the lipid interior. Compound 48/80 caused hemolysis after the transformation of erythrocytes into echinocytes.

Melittin and gramicidin S have also been reported to generate echinocytes at the concentrations causing hemolysis [29].

Aside from the erythrocyte shape changes studied here, the effects of antimicrobial peptides on bacterial membranes have been studied extensively by electron microscopy. When magainin II was used at a concentration where bacterial viability had decreased about 30%, blebs were observed on the surface of the bacteria [56]. Melittin also formed blebs on bacterial cells [57]. The formation of patches, structurally similar to blebs, is observed after treatment with various antimicrobial peptides [4,31]. The appearance of blebs or patches on bacterial membranes seemed to be similar to the change to echinocytes by human erythrocytes, as a result of the accumulation of peptides on the outer surface of the cell membranes. Hence, these results demonstrated that the breakdown of the biological membranes including erythrocytes and bacteria proceeded as proposed by the carpet model [4,31], accompanied by the accumulation of agents at the outer surface.

5. Conclusion

In this study, histamine-releasing agents induced K^+ efflux from mast cells, dependent on their ability to cause K^+ efflux from bacteria. The present results explained how the membrane permeabilization of mast cells by histamine-releasing agents correlated with their antimicrobial abilities. Furthermore, we observed that mastoparans changed the normal discoid shape of human erythrocytes to a crenated form at the concentrations causing hemolysis. An experiment using compound 48/80 and its components revealed that increasing the cationic charge changed the erythrocytes into echinocytes. Such changes correlated with blebs or patches formed on bacterial membranes, as a result of the

accumulation of peptides at the outer surface of the cell membranes. The present results supported that amphiphilic cationic peptides induced the breakdown of biological membranes including erythrocytes as proposed by the carpet model.

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Table 1

Peptides used.

Peptide	Sequence[a]
HR1	I-N-L-**K**-A-I-A-A-L-V-**K**-**K**-V-L-NH_2
Polistes mastoparan	V-**D**-W-**K**-**K**-I-G-Q-H-I-L-S-V-L-NH_2
Vespula mastoparan	I-N-L-**K**-A-L-A-A-L-A-**K**-**K**-I-L-NH_2
Granuliberin R	F-G-F-L-P-I-Y-**R**-**R**-P-A-S-NH_2
MCD peptide	I-**K**-C-N-C-**K**-**R**-H-V-I-**K**-P-H-I-C-**R**-**K**-I-C-G-**K**-N-NH_2
Magainin I	G-I-G-**K**-F-L-H-S-A-G-**K**-F-G-**K**-A-F-V-G-**E**-I-M-**K**-S
Magainin II	G-I-G-**K**-F-L-H-S-A-**K**-**K**-F-G-**K**-A-F-V-G-**E**-I-M-N-S
Melittin	G-I-G-A-V-L-**K**-V-L-T-T-G-L-P-A-L-I-S-W-I-**K**-**R**-**K**-**R**-Q-Q-NH_2
Gramicidin S	*cyclo*(V**O**LF^dPV**O**LF^dP)

[a] Amino acids are labeled according to the one-letter code with the following additions: O = ornithine, F^d = D-phenylalanine. Block letters indicate basic and acidic amino acids. Acidic amino acids, aspartic acid (D) and glutamic acid (E), contained in *Polistes* mastoparan and magainins, respectively. Disulfide linkages are shown by solid lines.

Table 2

ED_{50} values (concentrations giving 50% K^+ efflux, cell viability, hemolysis and histamine release) of mastoparans and related histamine-releasing compounds. [a]

	S. aureus cells		Human erythrocytes		Rat peritoneal mast cells	
	K^+ efflux	Cell viability	K^+ efflux	Hemolysis	K^+ efflux	Histamine release
HR1	2	2	50	50–100	10	5
Polistes mastoparan	5	5	50	100–200	10	2–5
Vespula mastoparan	2–5	2–5	50	50–100	10–20	5–10
Granuliberin R	500	500	>500	>500	200–500	10
MCD peptide	>50	>50	>200	>200	>50	0.05
Compound 48/80	100	50–100	>500	>500	10–20	0.5
Magainin I	10	10	>200	>200	20	10–20
Magainin II	5–10	5–10	>200	>200	20	10–20
Melittin	0.2–0.5	0.2–0.5	0.2–0.5	0.2–0.5	1–2	0.5
Gramicidin S	2–5	2–5	10	10	20	10–20

[a] ED_{50} values are shown in μM, except for compound 48/80 (μg/ml).

Figure Legends

Fig. 1. Structure of (a) compound 48/80 and its components; (b) monomer, (c) dimer, and (d) trimer.

Fig. 2. (a) Calibration graph for the K^+-selective electrode in a K^+-free buffer comprising 0.154 M NaCl, 0.9 mM $CaCl_2$, 5.6 mM glucose, 0.01% bovine serum albumin, and 5 mM Hepes−NaOH (pH 7.4). (b) The efflux of K^+ from mast cells induced by HR1. The mast cell suspension (30 μl, final cell concentration of 8×10^5 cells/ml) was added to 170 μl of the K^+-free buffer at 20°C at the time indicated by the first arrow. The second arrow indicates when 1 μl of 2 mM HR1 (final concentration, 10 μM) was added. The third arrow indicates when melittin (final concentration, 50 μM) was added to disrupt the cytoplasmic membrane of mast cells to determine the total amount of K^+ in the cells.

Fig. 3. The dose-response graphs of K^+ efflux (○), cell viability (●), hemolysis (×), and histamine release (▲) in (a) *S. aureus* cells, (b) erythrocytes, and (c) mast cells induced by HR1. *S. aureus* cells were suspended in 100 mM choline chloride and 50 mM Mops−Tris (pH 7.2) at 5×10^9 cells/ml. Human erythrocytes were suspended in 0.15 M NaCl and 5 mM Hepes−NaOH (pH 7.4) at 1×10^8 cells/ml. Mast cells were suspended in a K^+-free buffer solution comprising 0.154 M NaCl, 0.9 mM $CaCl_2$, 5.6 mM glucose, 0.01% bovine serum albumin, and 5 mM Hepes−NaOH (pH 7.4) at 8×10^5 cells/ml. *S. aureus* cells and erythrocytes were incubated with HR1 at 37°C for 30 min. The amounts of K^+ and histamine released from mast cells were determined after cells were incubated with HR1 at 20°C for 5 min.

Fig. 4. Granuliberin R-induced K^+ efflux, cell viability, hemolysis, and histamine release in (a) *S. aureus* cells, (b) erythrocytes, and (c) mast cells. The symbols used are the same as those in Fig. 3.

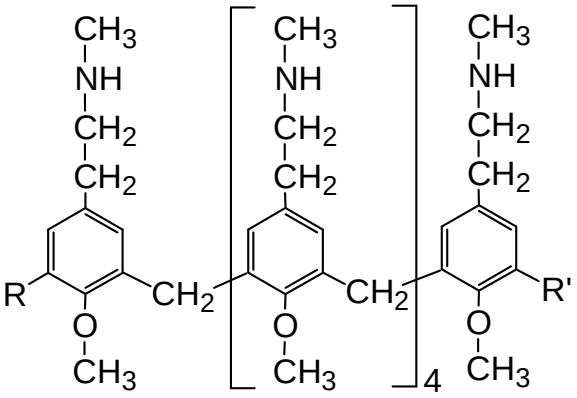
Fig. 5. Compound 48/80-induced K^+ efflux, cell viability, hemolysis, and histamine release in (a) *S. aureus* cells, (b) erythrocytes, and (c) mast cells. The symbols used are the same as those in Fig. 3.

Fig. 6. Changes in the morphology of human erythrocytes. (a) Intact cells. Cells were incubated with (b) HR1 (50 μM), (c) *Polistes* mastoparan (100 μM), or (d) *Vespula* mastoparan (50 μM) at 37°C for 30 min.

Fig. 7. Changes in the morphology of human erythrocytes induced by compound 48 and its constituents. Cells were incubated at 200 μg/ml with (a) monomer, (b) dimer, (c) trimer, or (d) compound 48/80 at 37°C for 30 min.

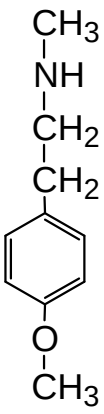
Fig. 8. Changes in a morphological index as a function of the concentration of compound 48 and its constituents. Erythrocytes were incubated with monomer (×), dimer (○), trimer (Δ), or compound 48/80 (●) at 37°C for 30 min.

Fig. 1



R, R' = H or CH_2OH

(a)



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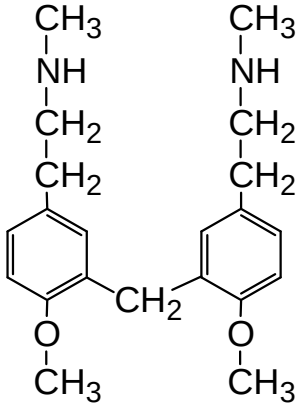

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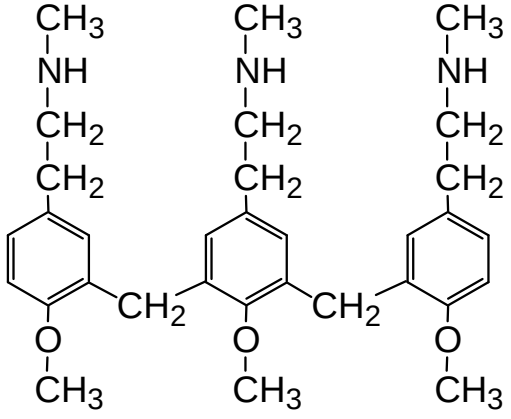


(d)

Fig. 2

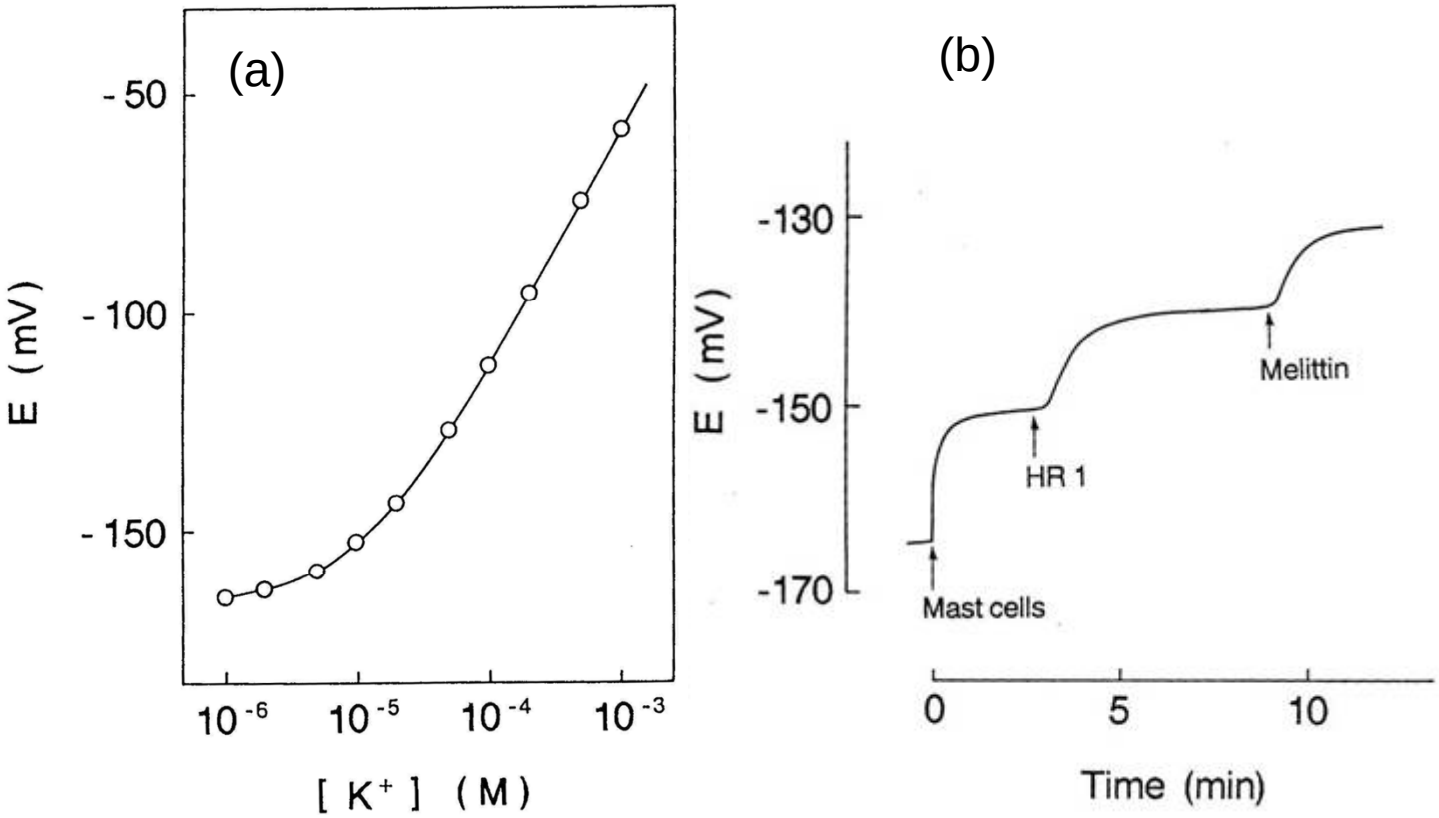

(a)
- 50
- 100
- 150
E (mV)
10^{-6}
10^{-5}
10^{-4}
10^{-3}
[K^+] (M)
(b)
-130
-150
-170
E (mV)
Melittin
HR 1
Mast cells
0
5
10
Time (min)

Fig. 3

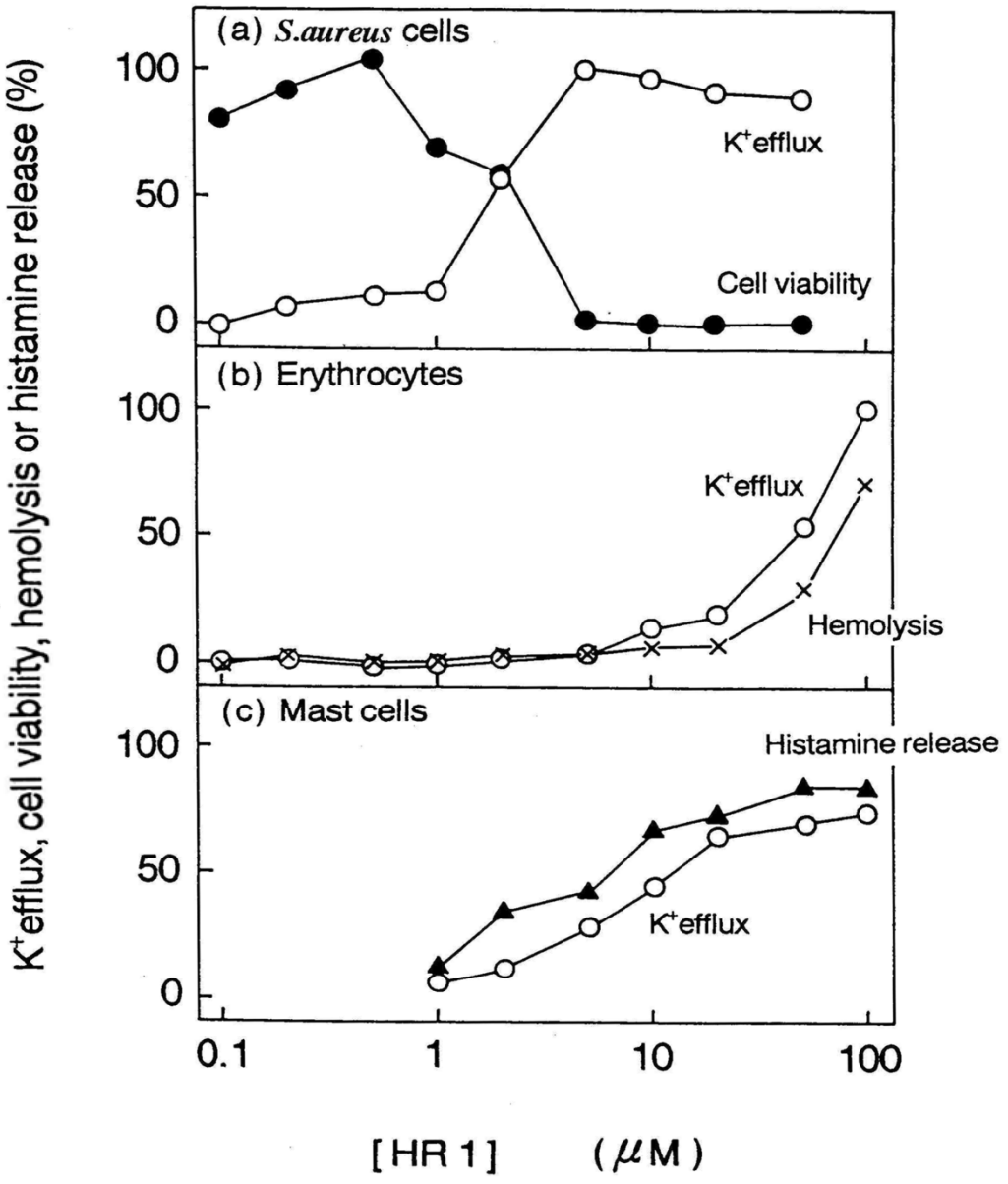

(a) S.aureus cells
100
50
0
K+efflux
Cell viability
(b) Erythrocytes
100
50
0
K+efflux
Hemolysis
(c) Mast cells
100
50
0
Histamine release
K+efflux
0.1
1
10
100
K+efflux, cell viability, hemolysis or histamine release (%)
[HR 1] (μM)

Fig. 4

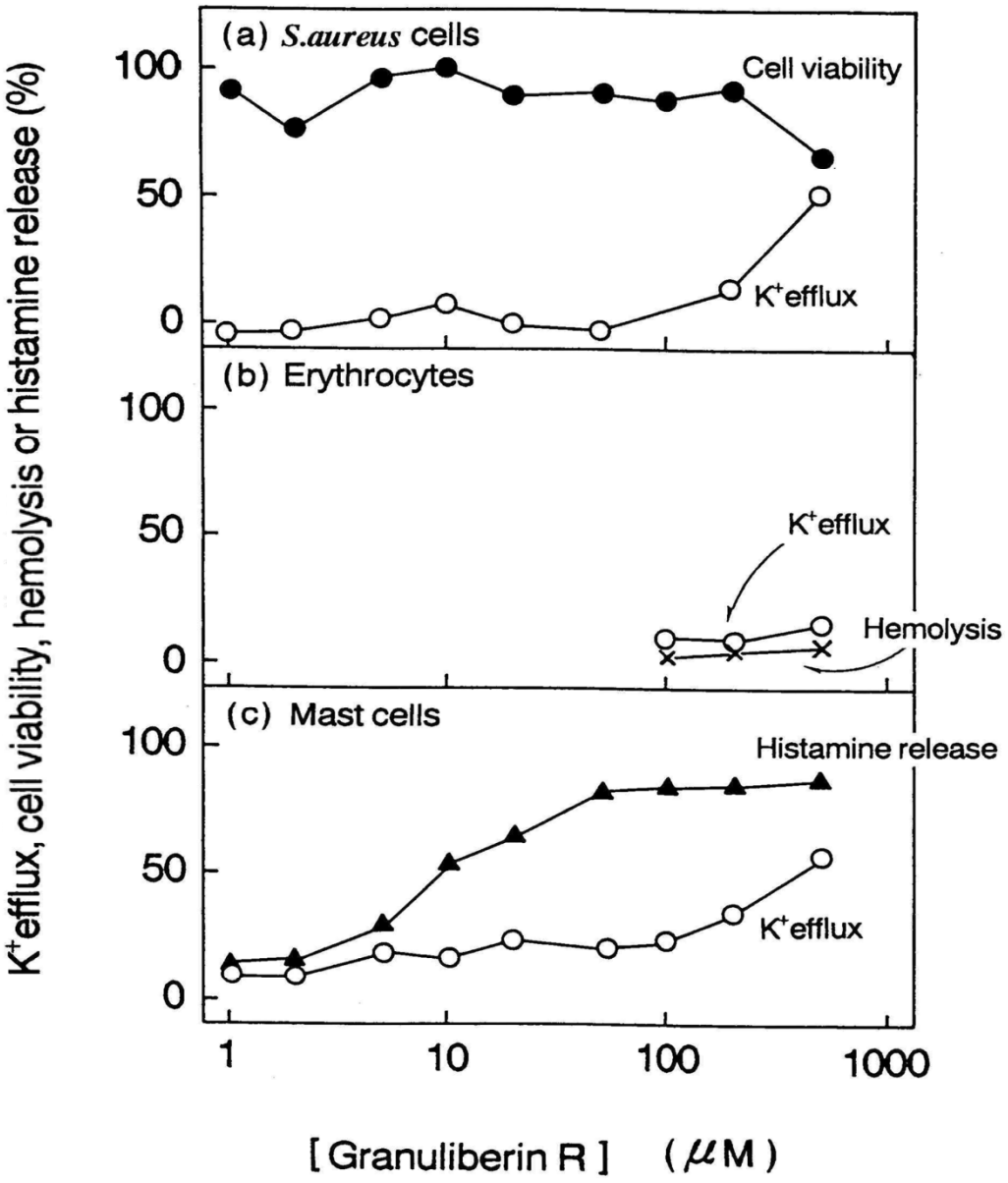

(a) S.aureus cells
Cell viability
K+efflux
(b) Erythrocytes
K+efflux
Hemolysis
(c) Mast cells
Histamine release
K+efflux
100
50
0
1
10
100
1000
K+efflux, cell viability, hemolysis or histamine release (%)
[Granuliberin R] (μM)

Fig. 5

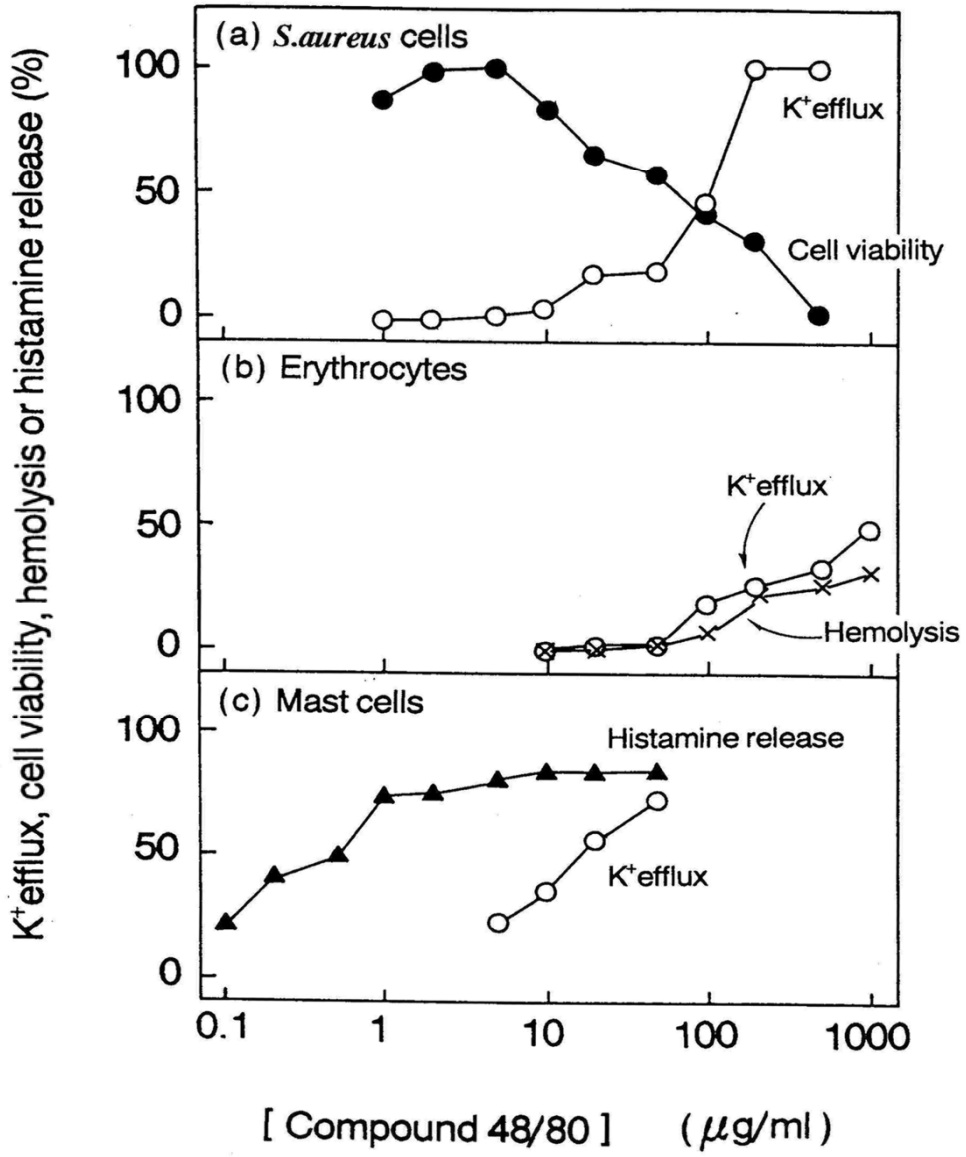

(a) S.aureus cells
100
50
0
K+efflux
Cell viability
(b) Erythrocytes
100
50
0
K+efflux
Hemolysis
(c) Mast cells
100
50
0
Histamine release
K+efflux
0.1
1
10
100
1000
K+efflux, cell viability, hemolysis or histamine release (%)
[Compound 48/80] (μg/ml)

Fig. 6

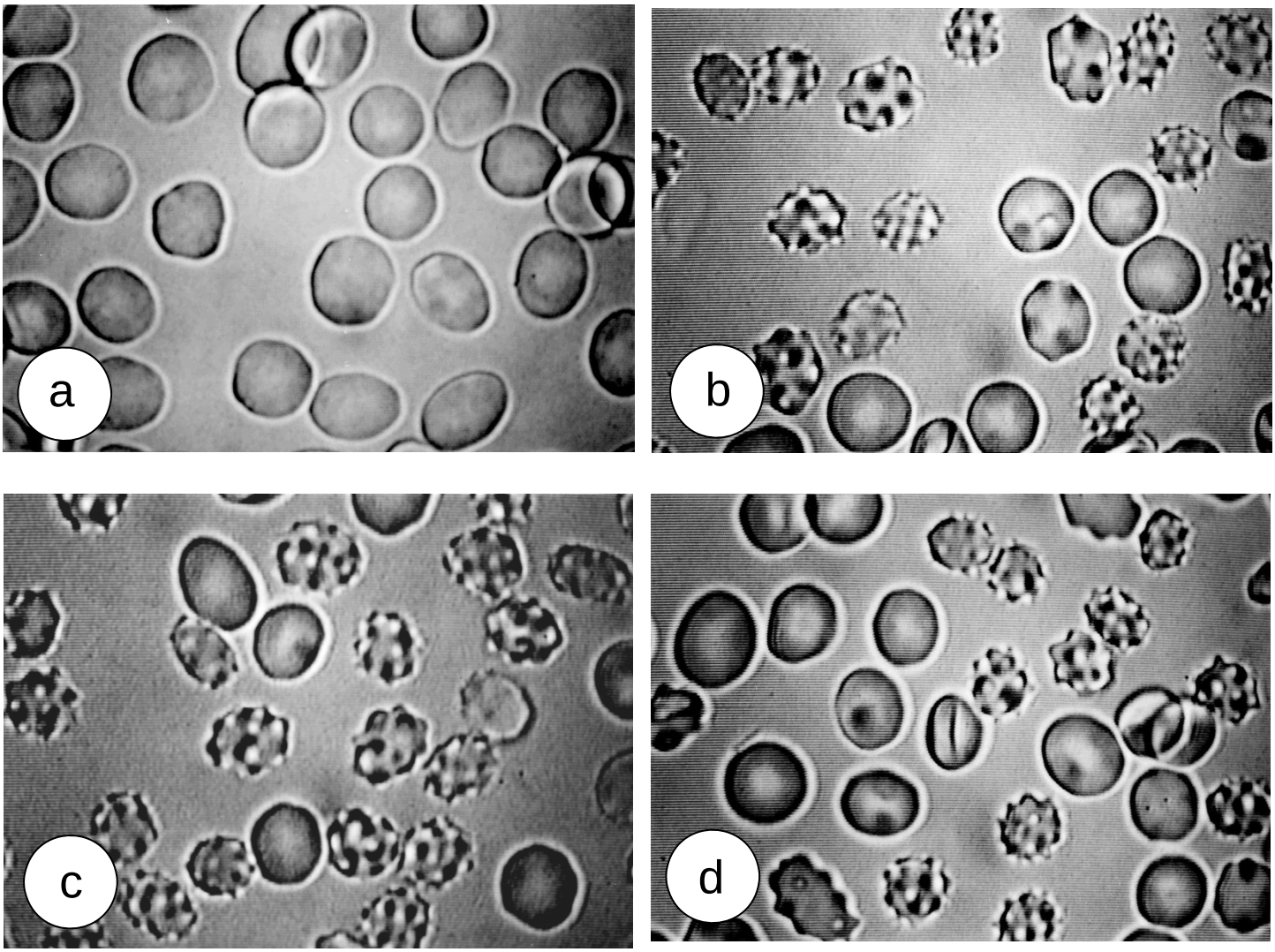


Fig. 7

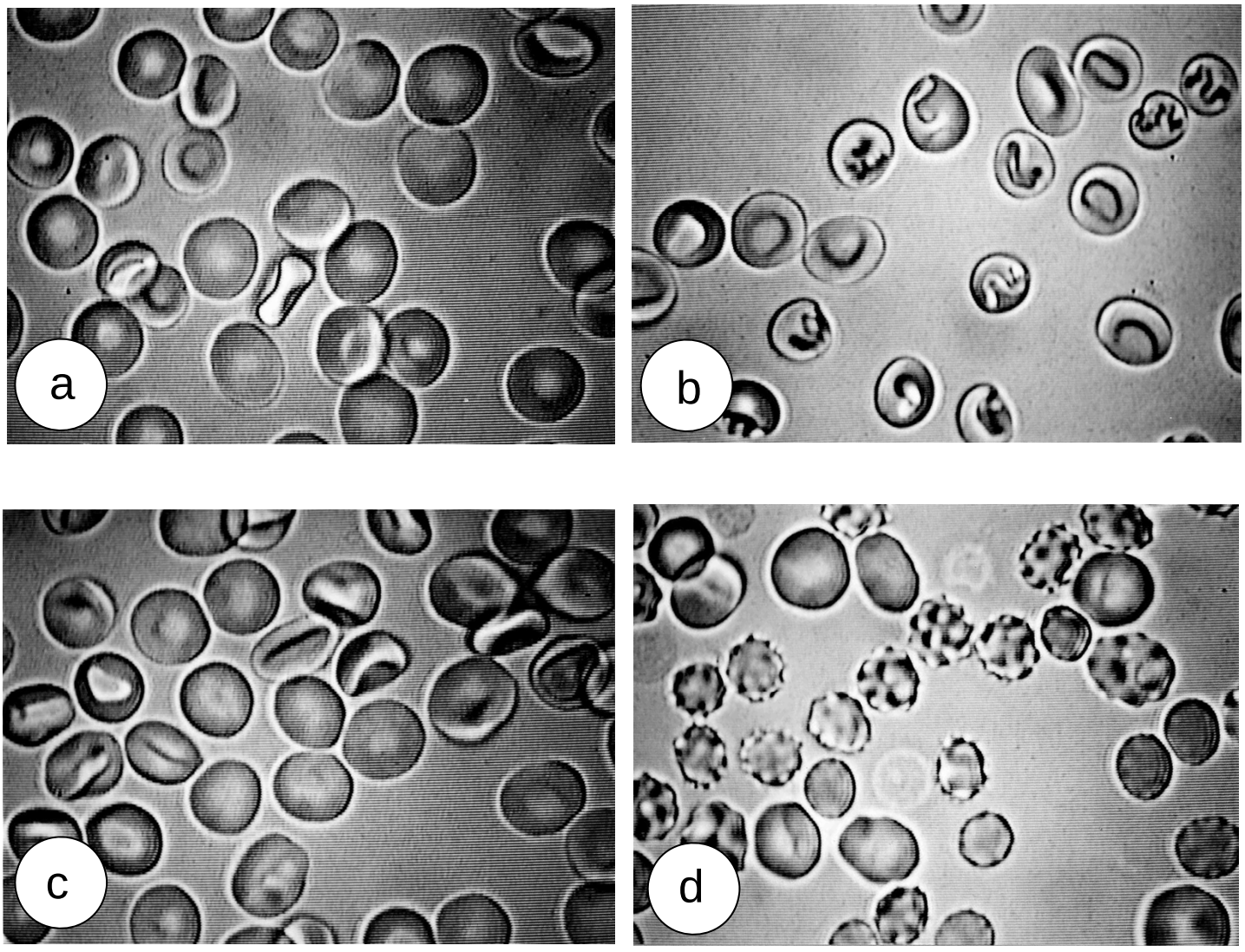
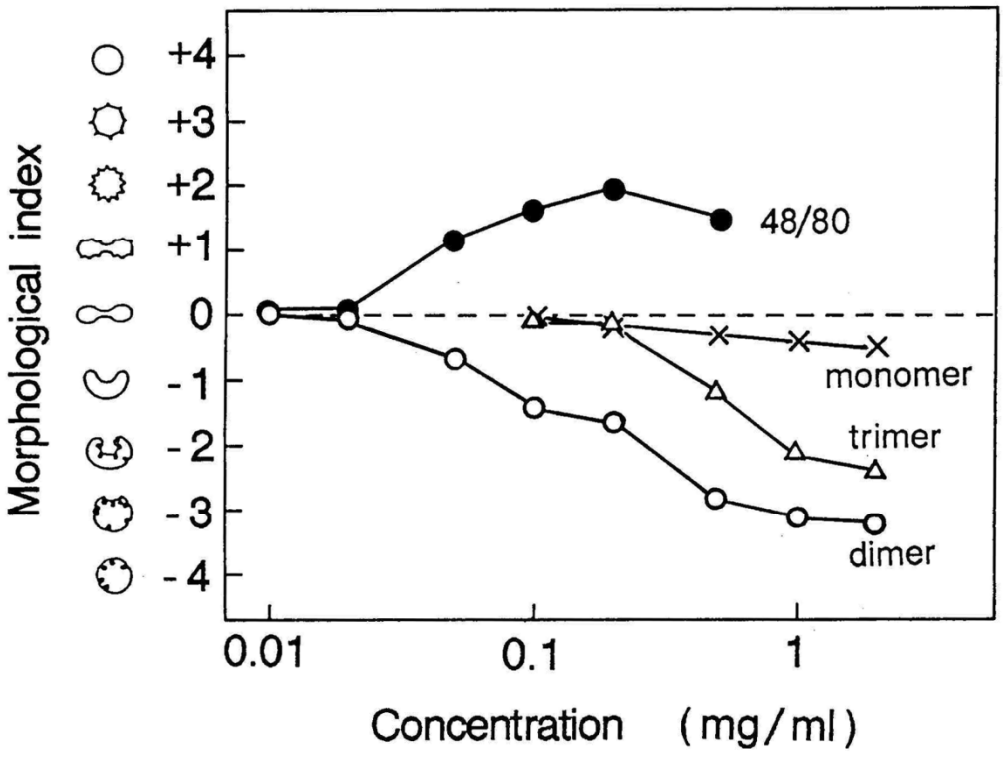


Fig. 8


Morphological index
+4
+3
+2
+1
0
-1
-2
-3
-4
48/80
monomer
trimer
dimer
0.01
0.1
1
Concentration (mg/ml)