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Short Communication

ALKALI CATION TRANSPORT THROUGH LIPOSOMES BY THE ANTIMICROBIAL FUSAFUNGINE AND ITS CONSTITUTIVE ENNIATINS

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Abstract—Fusafungine is a peptide antibiotic mixture composed of several enniatins and active against Gram-positive bacteria. Ionophoric properties of fusafungine have been studied in liposomes by measuring proton-cation exchange by both fluorescence and ^{31}P-nuclear magnetic resonance (NMR) and have been compared to those of its constituent enniatin peptides. Fusafungine, as well as enniatins, transport cations through a mobile carrier mechanism selective for K^+ *vs.* Na^+ and involving two antibiotic molecules. The transport efficiencies of the various enniatins appear to be related to their hydrophobicity, in agreement with a previously proposed "sandwich" transport model. The ionophoric properties of crude fusafungine may be involved in its antibiotic action and its local therapeutic properties.

Key words: fusafungine; enniatins; liposomes; cation transport; antibiotics

Fusafungine is an antibiotic extracted from the fungus *Fusarium Laetarium WR strain 437*, which displays antimicrobial activities against several Gram-positive bacteria and cocci [1]. The antibiotic is used locally for treatment of respiratory pathology [2]. Fusafungine is a defined mixture of enniatin cyclohexadepsipeptides made up of alternating D-α-hydroxyvaleric and L-N-methylamino acid residues [3]. Individual enniatins have been shown to possess ionophoric activities in model membranes. Monovalent cation transport by several enniatins has been studied in black-lipid membranes and has been suggested to occur through a mobile carrier mechanism [4, 5]. However, an uncertainty exists concerning the stoichiometry of the enniatin/cation permeating complex, which has been reported to be 1:1 [5] or 2:1 [6]. It is not known whether the ionophoric activity of enniatins is involved in the antibiotic action of fusafungine. This raises the question of to which extent this ionophoric activity is preserved in the crude mixture. This feature clearly depends on the transport mechanism and, especially, on its stoichiometry. Here, we studied in detail the ionophoric activity of crude fusafungine in comparison with those of individual enniatins with regard to both mechanism and stoichiometry. For this purpose, we measured cation-proton exchange in unilamellar liposomes by both fluorescence changes of an entrapped pH sensitive probe and ^{31}P-NMR§ of internal inorganic phosphate. Both methods have been used recently to study the ionophoric activity of other antibiotics [7, 8].

Materials and Methods

Chemicals. Phosphatidylcholine and phosphatidic acid from egg yolk were purchased from Avanti Polar Lipids. Pyranine was from Eastman Kodak. Valinomycin and FCCP were from Sigma and enniatins A, A1, B, and B1 from Interchim. Fusafungine was a generous gift of Laboratoires Servier (Orleans, France). Fusafungine is composed of enniatins A, A1, B, and B1 in 2:16:40:42 mole ratio [1]. Molar concentrations of fusafungine were calculated using a weighted average molecular weight of 650.

Fluorescence measurement. Fluorescence measurements were performed as described previously [7]. Unilamellar liposomes with a mean diameter of 150 nm were prepared by reverse phase evaporation using a 9:1 mixture of phosphatidylcholine and phosphatidic acid (mole-mole) in 20 mM KH_2PO_4-KOH, pH 7.1, 130 mM K_2SO_4 or 20 mM NaH_2PO_4, pH 7.1, 130 mM Na_2SO_4 containing 200 μM pyranine. External pyranine was removed by size-exclusion chromatography on a prepacked Sephadex G50 column (Pharmacia) in the same buffer. Liposomes eluted in the void volume and external pyranine was retained in the column. Liposomes were diluted in buffer at a lipid concentration of 0.5 mM in the fluorescence cuvette. The fact that 97% of the pyranine remained entrapped after such dilution (see Results) indicates that the integrity of the liposomes was not affected. Control experiments performed without pyranine indicated that the turbidity of the liposome suspension corresponded to 3% of the intensity measured with the fluorescence probe. A 0.5 unit pH gradient (outside acidic) was produced by adding concentrated H_2SO_4 to lower the external pH to 6.6. The necessary amount of H_2SO_4 was determined potentiometrically in separate experiments performed with identical sample conditions. Proton entry was measured from the fluorescence decay kinetics. Antibiotic-induced ion fluxes J_{net} were calculated according to Seigneuret and Rigaud [7] from the initial slopes of the fluorescence decrease and corrected for those obtained without antibiotics.

^{31}P-NMR experiments. Unilamellar liposomes were prepared at an initial phospholipid concentration of 62.5 mM in 75 mM KH_2PO_4, pH 6.2, 125 mM K_2SO_4, 1 mM EDTA, diluted 4 times in 200 mM K_2SO_4 and brought to pH 7.2 with KOH before addition of the antibiotics. 1 mM $MnCl_2$ was added to eliminate the external phosphate signal. ^{31}P-NMR experiments were recorded as described previously [8].

Results

Cation transport by fusafungine and enniatins. To study cation transport by fusafungine, a fluorescence assay based on the measurement of transmembrane proton-cation exchange [7]

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§ Abbreviations: pyranine; 8-hydroxy-1,3,6-pyrenetrisulfonate; FCCP, carbonyl cyanide p-(trifluoromethoxy)-phenylhydrazone; NMR, nuclear magnetic resonance.

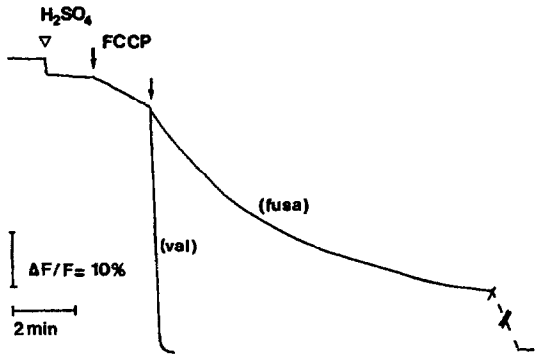

Fig. 1. Fluorescence response of pyranine entrapped into liposomes in 130 mM K_2SO_4, 20 mM KH_2PO_4-KOH, pH 7.0, after a 0.5 pH unit external pH pulse (indicated by open arrow). First solid arrow: addition of FCCP (1 μM); second solid arrow: addition of valinomycin (0.1 μM) or fusafungine (0.15 μM).

was used. Liposomes in K_2SO_4 medium containing entrapped pyranine, a nonpermeant pH-sensitive probe, were first submitted to a 0.5 unit pH gradient (inside acidic) and proton equilibration was monitored fluorimetrically. (Fig. 1). A fast ca. 3% decrease in fluorescence intensity was initially observed due to a small amount of residual external pyranine [7]. The slower subsequent fluorescence decrease represented the internal pH decrease associated with passive diffusion of protons into liposomes. Addition of the proton carrier FCCP accelerated the initial rate of proton equilibration by a factor of 3.6. In the presence of FCCP, proton movement is electrically limited by the diffusion of other permeant charged specie(s); here, the chosen alkali cation K^+ [7]. This was confirmed by the very fast equilibration that occurred upon further addition of the K^+ carrier valinomycin. Thus, in the presence of FCCP, the observed proton flux could be taken as a measure of monovalent cation permeability. Under such conditions, addition of fusafungine also accelerated proton equilibration. No effect was observed in the absence of FCCP, excluding proton transport by fusafungine (data not shown). This indicates that fusafungine acts as a K^+ ionophore. Its efficiency appears much lower than that of valinomycin (*i.e.* the half-times of proton equilibration are 3 s and 480 s for similar concentrations of the two antibiotics). Similar experiments performed in Na^+ medium showed that fusafungine also promotes Na^+ transport (see below).

Figure 2A shows double-logarithmic plots of initial cation flux *vs.* antibiotic concentration for crude fusafungine. For both K^+ and Na^+, a slope of 2 was found, indicating the involvement of a bimolecular complex. Figure 2A also allows a straightforward comparison of enniatin cation selectivity (*i.e.* 8 times faster ion fluxes were observed for K^+ as compared to Na^+ irrespective of antibiotic concentration).

The cation transport activity of individual constituant enniatins was also studied. As indicated in Fig. 2B, all 4 component enniatins induced K^+ transport in liposomes with a similar bimolecular stoechiometry. Significant differences were found in enniatin transport efficiencies, which were in the order A > A1 > B > B1.

Assessment of fusafungine transporter type by ^{31}P-NMR. To assess whether the bimolecular complex acts as a mobile carrier or as a pore, ^{31}P-NMR experiments were carried out. The chemical shift of inorganic phosphate entrapped in liposomes was used to measure internal pH and to monitor cation-proton exchange. The distribution of internal pH values among liposomes could be visualized during transport and allows for a nonambiguous determination of the molecular type of ionophore involved [8]. Liposomes were prepared at pH 6.2 in concentrated phosphate buffer (75 mM) and Mn^{2+} was added to suppress the external phosphate ^{31}P signal. A 1 unit pH gradient (inside acidic) was elicited before addition of FCCP and fusafungine (Fig. 3). Fusafungine was present at antibiotic molecule/liposome. Initially, the spectrum was composed by a single peak of internal phosphate at 0.77 ppm corresponding to an internal pH of 6.2. This internal phosphate signal progressively shifted due to proton-cation exchange by fusafungine. A constant chemical shift of 2.32 ppm was reached after 15 hr which corresponded to a final internal pH of 7.2 (*i.e.* complete dissipation of the pH gradient). Only one single peak, although transiently broadened, is observed during the whole kinetics, indicating that K^+/H^+ exchange occurred homogeneously and progressively among the whole liposome population in spite of the substoichiometric antibiotic concentration. This indicates that fusafungine exchanges between liposomes more rapidly than H^+ equilibration can occur. This behavior is characteristic of a mobile carrier-type transport and contrasts with a pore-type transport that would lead to rapid pH equilibration of individual liposomes and slower antibiotic exchange among liposomes yielding heterogenous populations in ^{31}P-NMR experiments [8].

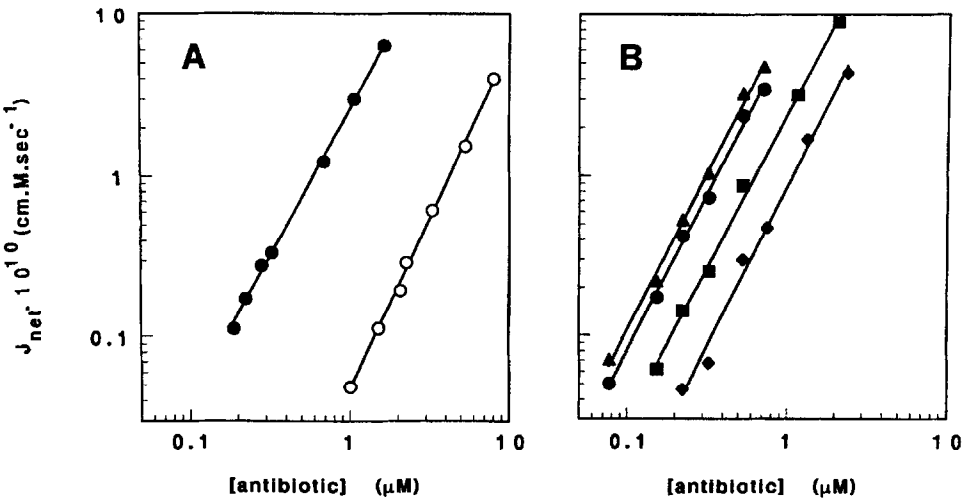

Fig. 2. Panel A: Effect of fusafungine concentration upon K^+ (●) and Na^+ (○) transport in liposomes. Proton fluxes were calculated for a pH gradient value of 0.25 pH units. Panel B: Effect of concentration upon K^+ transport for Enniatin A (▲), A1 (●), B1 (■) and B (♦).

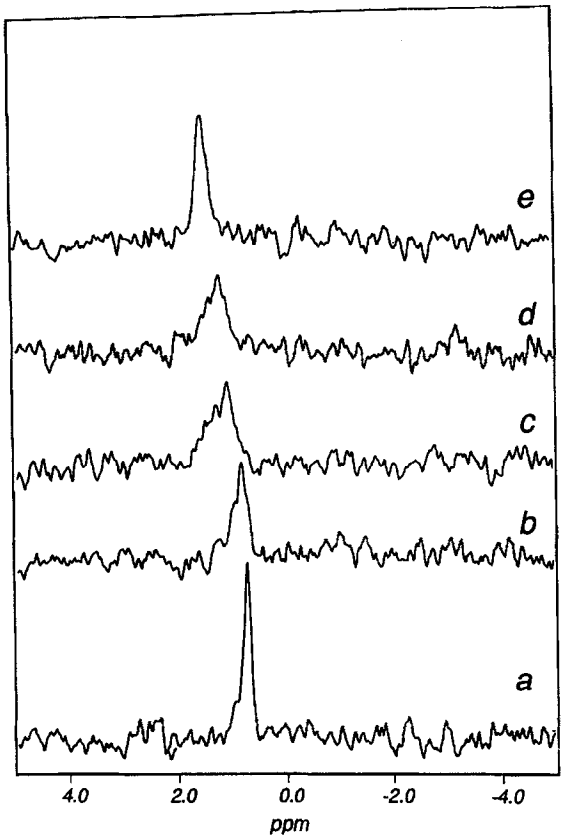

Fig. 3. ^{31}P-NMR spectra of intraliposomal inorganic phosphate after a 1 unit pH gradient in the presence of 1 μM FCCP and 50 nM fusafungine added at t = 0. Spectrum a was recorded before addition of the antibiotics. Spectra b, c, d, and e were recorded after incubation times of 1.3, 3.75, 5.3, and 15 hr.

Discussion

This study indicates that crude fusafungine possesses an ionophoric activity similar to that of individual enniatins. ^{31}P-NMR measurement indicates that fusafungine acts as a mobile carrier for monovalent cations. Fluorescence measurements show that this mobile carrier has a significant selectivity for K^+ compared to Na^+. The involvement of a K^+-selective mobile carrier has already been suggested for individual enniatins from black lipid membrane experiments and is confirmed here [4, 5]. Furthermore, it is shown that fusafungine, as well as individual enniatins, acts as a bimolecular complex. This confirms the suggestion that a 2:1 antibiotic-cation complex may be the translocating species [6]. This proposal was disputed in another study [5]. The fact that crude fusafungine can act as a cation ionophore suggests that this activity is involved in its antibiotic properties.

The transport efficiencies of individual enniatins have also been compared. All enniatin appears to act as a 2:1 complex with cations. Interestingly, higher transport rates are found for the more lipophilic enniatins (*i.e.* A (three N-methylisoleucine) > A1 (two N-methylisoleucine, one N-methylvaline) > B1 (one N-methylisoleucine, two N-methylvaline) > B (three N-methylvaline). These data are in agreement with the "sandwich" model of the cation-enniatin 2:1 complex in which the hydrophobic sidechains are externally located. An increase in the hydrophobicity of the complex is expected to decrease the energy barrier for permeation through the membrane [6]. This suggests that a way to increase the efficiency of these antibiotics would lie in the use of analogs with more hydrophobic sidechains.

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