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Pamamycin Inhibits Nucleoside and Inorganic Phosphate Transport in *Staphylococcus aureus**

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SUMMARY

Pamamycin is a stimulator of aerial mycelia formation in *Streptomyces alboniger* and a new antibiotic active against many microorganisms. Studies in *Staphylococcus aureus* showed that pamamycin inhibited the uptake of nucleosides and inorganic phosphate, as well as purine and pyrimidine bases. Under the same conditions, other cellular functions including protein and cell wall synthesis, amino acid uptake, glucose utilization and DNA and RNA polymerase activities were not affected. The existence of respiration-dependent transport systems for nucleosides in membrane vesicles of *S. aureus* also was demonstrated. Pamamycin inhibited membrane transport of all the nucleosides tested. The inhibition of the above membrane-associated functions was probably due to the membrane-binding property of this antibiotic.

INTRODUCTION

Several compounds have been isolated from streptomycetes which differentially affect aerial mycelia formation and antibiotic production (1,2). We recently described the isolation and properties of pamamycin, which stimulates aerial mycelia formation in the producing host, *Streptomyces alboniger* (3). Pamamycin is also a new family-type antibiotic active against many Gram-positive bacteria, *Mycobacteria* and fungi. We have chosen *Staphylococcus aureus* as a model organism to study the primary cellular functions affected by this antibiotic, hoping the results will help in the subsequent study of its molecular mechanism as a differentiation effector. We report here that pamamycin inhibits the uptake of nucleosides, purines, pyrimidines and inorganic phosphate. The inhibition of nucleoside uptake is due to a specific effect on transport.

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EXPERIMENTAL PROCEDURES

Pamamycin - The isolation, characterization and unit definition of the new antibiotic were previously described (3). Stocks were kept in toluene at 4°C. The antibiotic was assayed in a Me_2SO[1] solution and controls with Me_2SO alone were always included. In all the experiments reported below, Me_2SO had no effect.

Chemicals - The radioactive compounds used were [^{32}P]H_3PO_4, carrier free; [5,6-^{3}H]uridine, 40.8 Ci/mmol; [^{3}H-methyl]thymidine, 46 Ci/mmol; [4,5-^{3}H] isoleucine, 100 Ci/mmol, [2,3-^{3}H]valine, 17.4 Ci/mmol. All were from New England Nuclear except valine which was from Amersham. All other chemicals were from Sigma.

Growth of bacteria - *S. aureus* ATCC 6538P was grown in nutrient broth (Difco), brain heart infusion (BHI, Difco) broth or one-fifth strength BHI broth at 37°C with shaking. Working cultures were prepared by diluting (300x) an overnight culture into the same medium and growing to an A_{615} of 0.3 to 0.4.

Measurement of uptake - The working culture was kept on ice and used after 2 min warming at 37°C. Cells were transferred to flasks containing pamamycin (or Me_2SO) and the radioactive compound to be tested. Isotopes were diluted by varying levels of the respective nonradioactive compound present in the growth media. At intervals after incubation at 37°C, 1 ml was harvested under vacuum on a pre-wetted (with washing buffer) Millipore membrane filter (type HA, 0.45 μm) placed on a manifold filtration apparatus (Amicon, Lexington, Mass.). Cells were then washed with 5 x 1 ml of 50 mM potassium phosphate buffer, pH 7.3. The whole collection and washing processes were completed within 10 s. The filters were dried and radioactivities of ^{3}H were determined by liquid scintillation counting in Spectrafluor (Amersham) with an efficiency of 26%. The radioactivities of ^{32}P were determined by Cerenkov radiation with an efficiency of 36%. Total counts added in the control and pamamycin-treated culture were determined by spotting a small aliquots of the labeled culture onto the filter and counting as above. The results of the antibiotic-treated samples were normalized to those of control by the ratio of the two total counts added. All the results reported were those after subtracting the value of a blank which was determined by passing the same amount of total counts through the same amount of cells on the filter and processing as in the uptake experiment.

Preparation of membrane vesicles - Cultures in BHI broth were harvested at late exponential phase with A_{615} = 4.5-7.6. Membrane vesicles were prepared by the method of Konings *et al.* (4), except cells were resuspended in a minimal volume of 4.1 M NaCl and incubated with 50 units/ml lysostaphin (Sigma) for 2 h. One gram (wet weight) of cells yielded 7 mg membrane protein, determined by the method of Bradford (5) using bovine serum albumin as a standard.

Measurement of transport - The 400 μl reaction mixture contained 50 mM potassium phosphate with 5 mM potassium EDTA (pH 7.3), 10 mM $MgSO_4$, 10 μM radioactive compound with a specific activity of 0.5 Ci/mmol, 0.08 mg membrane protein and one of the electron donors. They were: 10 mM L-α-glycerophosphate (dicyclohexylammonium salt), 20 mM L-lactate (lithium salt), 0.1 mM PMS plus 10 mM ASC. Reactions were carried out at 25°C with vigorous shaking. Vesicles were first equilibrated at 25°C in 360 μl buffer and $MgSO_4$ for 30 s. Pamamycin in 2 μl Me_2SO was added and the mixture was incubated for 2 min. The reaction was initiated by adding a mixture of radioactive compound and the electron donor. In the case of the PMS/ASC system, the mixture of labeled compound and ascorbate was added 30 s after the PMS addition. At the desired time, the reaction was

[1]The abbreviations used are: Me_2SO, dimethylsulfoxide; PAM, pamamycin; PMS, phenazine methosulfate; ASC, sodium ascorbate; αGP, L-α-glycerophosphate.

stopped by adding 2 ml cold 0.1 M LiCl, filtering through a 0.45 μm Amicon microporous filter under vacuum and washing with 2 x 2 ml LiCl. The whole process was completed in less than 10 s. The filters were dried and counted as above. Zero minute transport activities were obtained by adding LiCl before the addition of labeled compound and the electron donor and had a value of 200-400 cpm. All results reported here are after subtracting the zero minute values.

RESULTS

Pamamycin inhibits uptake of nucleosides, purine and pyrimidine bases - Pamamycin was bacteriostatic against *S. aureus* in BHI broth, 0.14 U/ml inhibiting growth 40%. It became bactericidal when higher levels were used (>0.5 U/ml). Studies of the effect on macromolecular synthesis showed pamamycin inhibited the incorporation of thymidine and uridine into DNA and RNA. Under the same conditions, the antibiotic had no effect on protein and cell wall synthesis. Since the incorporation of exogeneous precursors into macromolecules involved transport of the precursors into the cell, the effect of pamamycin on uptake of nucleosides was investigated in order to have a rough measurement of the effect on transport. Uptake here was defined as total cellular-associated counts after cells are separated from the labeled nucleosides in the incubation medium. The results in Fig. 1a show that the initial uptake of uridine was inhibited by pamamycin. Similar inhibition was seen of the uptake of thymidine, cytidine, adenosine and deoxyuridine, as well as of the free bases, adenine and uracil. Under the same conditions, the uptake of valine (Fig. 1b) and other amino acids,

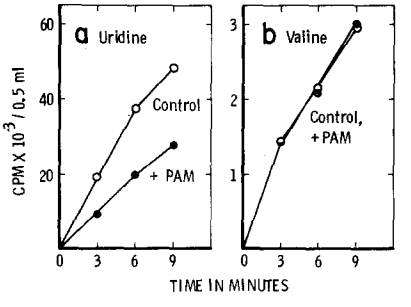

Fig. 1 Differential inhibition by pamamycin of uridine and valine uptake in *S. aureus*. Cells were grown in one-fifth strength BHI broth to A_{615} = 0.37. Portions of the culture were transferred to flasks containing Me_2SO (o——o, 0.1%) or pamamycin (●——●, 0.14 U/ml) and [^{3}H]uridine (6.5 x 10^5 cpm/ml) (a) or [^{3}H]valine (4.2 x 10^6 cpm/ml) (b). Total cellular-associated counts were measured as described in Experimental Procedures.

2-deoxyglucose and Mn^{+2} and the utilization of glucose (measured by initial uptake) was not affected.

Subsequent studies showed the accumulation of uridine into the TCA-soluble pool was inhibited much more than the incorporation into RNA. Also, uridine uptake was inhibited identically by pamamycin under conditions where RNA synthesis was inhibited 90% by the addition of rifampicin. In addition, the activities of DNA and RNA polymerases were not affected by very high levels of pamamycin. Therefore, pamamycin appeared to inhibit the transport of nucleic acid precursors.

Pamamycin inhibits nucleoside transport in membrane vesicles of *S. aureus* - Direct evidence for inhibition of transport was obtained by studies of nucleoside transport in isolated membrane vesicles prepared according to the procedure of Konings *et al.* (4). Such membrane vesicles from *S. aureus* were found to accumulate uridine in the presence of three electron donor systems (Fig. 2). The accumulated uridine was not changed chemically as judged by TLC after extraction. The αGP-dependent transport was the least efficient one among the three systems when vesicles were prepared from cells grown in BHI broth where glucose was the major carbon source. This phenomenon was also observed by others during the study of amino acid transport in membrane vesicles of *S. aureus* (6). The results in Figs. 2 and 3 show that, in addition, pamamycin inhibited uridine transport in all three systems. The membrane transport of

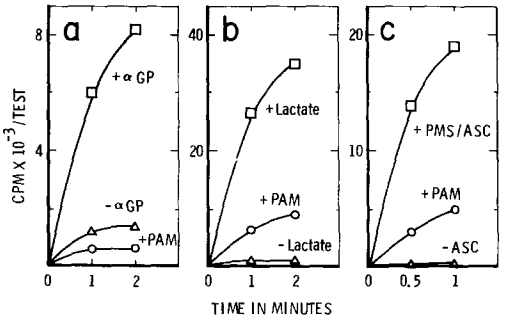

Fig. 2. Inhibition of uridine transport by pamamycin in membrane vesicles of *S. aureus*. Transport was measured in the presence (□——□) or absence (Δ——Δ) of electron donors: αGP (a), L-lactate (b) and PMS/ASC (c). Pamamycin (O——O) was added in the complete reaction mixture to 1 U/ml (a and b) or to 0.25 U/ml (c). See Experimental Procedures for details.

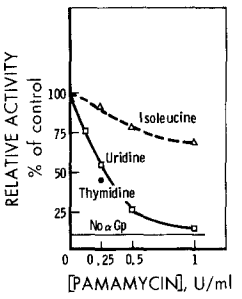

Fig. 3. Effect of pamamycin on the transport of uridine, thymidine and isoleucine in membrane vesicles of *S. aureus*. Relative transport activities (1 min results) of isoleucine (Δ), uridine (□) and thymidine (●) were determined in the presence of varying concentrations of pamamycin. 100% isoleucine transport = 3141 cpm, 100% uridine transport = 5247 cpm, 100% uridine transport = 4829 cpm. αGP was used as the electron donor. See Experimental Procedures for details.

thymidine (Fig. 3), cytidine and adenosine also was inhibited by pamamycin in the three electron donor systems. The degree of inhibition (50-70%) and pamamycin concentration used (0.25 U/ml) were comparable to those of uptake studies in whole cells. Thus, it is very clear that there exists a respiration-dependent active transport system for all the nucleosides tested in membrane vesicles of *S. aureus*, and pamamycin inhibits the transport process. For reasons unknown, these vesicles did not have transport activity for adenine and uracil. Several amino acids (isoleucine, leucine, phenylalanine) also were actively transported in these vesicle preparations. Inhibition of amino acid transport by pamamycin was less sensitive than nucleoside transport (Fig. 3), but significantly higher than that found in uptake studies with whole cells.

Pamamycin inhibits uptake of inorganic phosphate - The growth of *S. aureus* in a synthetic medium without nucleosides or free bases was still sensitive to pamamycin. This antibiotic, therefore, must inhibit growth by a mechanism other than the inhibition of nucleoside transport. In an extension of these studies, one possibility has been found to explain the growth inhibition-namely, that pamamycin inhibits the uptake of inorganic phosphate in *S. aureus*. The uptake of $^{32}PO_4$ was measured in cells grown in nutrient broth because of the low phosphate concentration in this medium. *S. aureus* was more sensitive to pamamycin in this medium than in BHI broth-0.075 U/ml antibiotic inhibited growth 50%

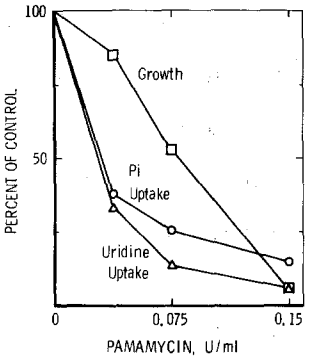

Fig. 4. Effect of pamamycin on the growth and uptake of inorganic phosphate and uridine in *S. aureus*. An exponentially growing culture (A_{615} = 0.34) in nutrient broth was divided into 3 portions to measure the growth and uptake. The slopes of the plots of A_{615} vs. time were used to determine the growth rates. The 2 min results of phosphate and uridine uptake studies described in the Experimental Procedures were used here. Both uptake activities were linear with time for at least 6 min. Total radioactivities used were 3.4×10^6 cpm/ml for $^{32}PO_4$ and 4.9×10^5 cpm/ml for [^{3}H]uridine. □——□: growth rate, o——o: inorganic phosphate uptake, Δ——Δ: uridine uptake. 100% growth = 0.118 A_{615}/10 min. 100% Pi uptake = 5419 cpm/ml. 100% uridine uptake = 9683 cpm/ml.

(Fig. 4). The Pi uptake was as sensitive to pamamycin as uridine uptake and both were much more sensitive than growth (Fig. 4). For example, at 0.0375 U/ml, growth was 85% of the control 10 min after the addition of pamamycin while the uptake of phosphate and uridine decreased to about 40% of the control in 2 min.

DISCUSSION

Pamamycin is a highly saturated alicyclic compound ($C_{36}H_{63}NO_7$) which belongs to a new family-type of antibiotics. This antibiotic was found to bind tightly to cytoplasmic membranes of *S. aureus*, *Mycobacterium phlei* and *Escherichia coli*. Thus, it is not unexpected that pamamycin inhibits membrane-associated functions. Pamamycin is the first antibiotic reported to inhibit inorganic phosphate uptake in bacteria. Since the initial uptake is inhibited, pamamycin seems to inhibit the transport process. Although transport of inorganic phosphate has been reported in membrane vesicles of *E. coli* (7), we could not detect such activity in our preparations.

Our results have demonstrated that pamamycin inhibits nucleoside, purine and pyrimidine uptake and nucleoside transport. Membrane vesicle transport of

nucleosides has been described in *E. coli* (8-10) and in *Salmonella typhimurium* (11). Here we have described nucleoside transport in membrane vesicles of *S. aureus*. We have not studied the detailed mechanism of this transport system, but it seems that the group translocation mechanism described in *Salmonella typhimurium* is not involved because uridine is transported into vesicles chemically unchanged. Showdomycin, a nucleoside analog, inhibits nucleoside transport by a competition mechanism (8,9). The mechanism of pamamycin inhibition is unknown. However, membrane bound αGP dehydrogenase and NADH oxidase activities were not affected. Also, the lack of inhibition under equivalent conditions of amino acid, 2-deoxyglucose and Mn^{+2} uptake, as well as of glucose utilization, suggests that pamamycin does not act by a primary effect on the protonmotive force.

We are now extending these studies to understand the mechanism of stimulation of aerial mycelia formation in *Streptomyces alboniger*. It is of interest that both the uptake of uridine and phosphate are inhibited by pamamycin in this organism.

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