STUDIES ON THE IONOPHOROUS ANTIBIOTICS. XII[1)]

EFFECTS OF IONOPHORE LYSOCELLIN ON CATION DISTRIBUTION AND RESPIRATION IN MITOCHONDRIA

Mitsuaki Mitani, Mitsuo Koenuma+ and Noboru Ōtake*

Institute of Applied Microbiology, University of Tokyo
Bunkyo-ku, Tokyo 113, Japan

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The effects of the ionophore lysocellin on the movements of Ca^{2+}, Mg^{2+} and alkali metal cations and its effect on energy utilization by rat liver mitochondria have been investigated. At a concentration of 0.05 μM, lysocellin induced dissociation of membrane-bound calcium, and an apparent steady state was established across the inner membrane between energy-linked calcium accumulation and the ionophore-induced depletion of calcium. No detectable efflux of intramitochondrial Ca^{2+} and Mg^{2+} was induced by 0.05 μM lysocellin, but the uptake of exogenously added calcium was significantly inhibited. The ionophore augmented Mg^{2+} release from mitochondria induced by Ca^{2+} addition and also caused rapid release of K^+ from mitochondria preloaded with K^+ by valinomycin or monazomycin. High levels (0.5 ~ 10 μM) of lysocellin caused massive depletion of endogenous Ca^{2+}, Mg^{2+} and K^+ from mitochondria, resulting in disruption of mitochondrial functions including release of state 4 respiration, stimulation of ATPase and inhibition of ADP- or DNP-stimulated respiration. Structure-activity studies with chemically modified compounds of lysocellin indicated the important role of terminal carboxylic acid and C_{21} hydroxyl function in the activity of the ionophore, and there is a good correlation between the effect of lysocellin on mitochondrial cation movements and its ability to complex with cations determined in an organic solvent-water two-phase partition system.

Lysocellin is a monocarboxylic antibiotic produced by *Streptomyces cacaoi,* whose structural elucidation is established by a three-dimensional X-ray analysis[2)]. Cation binding and transport studies using organic solvent barrier systems[3)] show that lysocellin is an ionophore with a broad cation selectivity which is able to transport mono- and divalent metal cations as well as biogenic amines. We previously[4)] reported that the ionophores lysocellin, etheromycin (CP-38295) and lasalocid A caused platelet aggregation as a result of simultaneous secretion of stored serotonin and efflux of intracellular calcium, indicating a function of these antibiotics as mobile carriers of the cations and amines across the biological membrane.

It has been postulated that by interacting with cyclic nucleotides, the intracellular calcium plays an important role in regulating cellular excitation mechanisms such as stimulus-secretion and stimulus-contraction coupling processes[5,6)]. The divalent cation ionophores A 23187 and lasalocid A (X-537A), which render membranes permeable to calcium, have recently served as useful tools for understanding these cellular systems[7,8)]. Because mitochondria are believed to be a major organelle controlling cytosolic calcium levels[5,6)], studying the effects of ionophores on isolated mitochondria may enable us to understand the action of the ionophores at the cellular level. In the present study, we show the effects of lysocellin on cation movements and respiration in mitochondria. Using chemically modified com-

* To whom correspondence should be directed.
+ Present address: Shionogi Research Laboratory, Shionogi & Co. Ltd., Fukushima-ku, Osaka 553, Japan

pounds of lysocellin, we also discuss the structure-activity correlation of the ionophores.

Materials and Methods

Chemicals. The antibiotics used were obtained from the following sources; Lysocellin and its chemically modified compounds, monazomycin and nigericin were the stock samples in our laboratory. Lasalocid A was a generous gift from Dr. J. G. WHITNEY (Eli Lilly and Co., Indianapolis, Ind., U.S.A.). Chlorotetracycline was purchased from Calbiochem, and valinomycin from P-L. Biochemicals Inc., Milwaukee, Wis. U.S.A.

Mitochondria. Mitochondria were prepared from livers of male rats as described by JOHNSON and LARDY[9].

Measurement of Ca^{2+} and Mg^{2+} movements by mitochondria. The intramitochondrial contents of Ca^{2+} and Mg^{2+} were determined by atomic absorption analysis or by the fluorescence chelate probe chlorotetracycline technique of CASWELL.[10] Mitochondria were prepared for atomic absorption by rapidly sedimenting in a centrifuge and subsequently dispersing the pellet with 1% desoxycholate in distilled water.

Measurements of oxygen consumption, light-scattering and potassium movement in mitochondria. Respiration was measured polarographically with a Clark-type oxygen electrode. Mitochondrial swelling was monitored at 515 nm by light-scattering changes with an Hitachi 333 recording spectrophotometer. The concentration of K^+ was monitored with an Orion K^+-specific electrode 92–19.

Measurement of adenosine triphosphatase activity in mitochondria. Adenosine triphosphatase (ATPase) activity was determined by measuring inorganic phosphate release as described previously[11].

Results

Effects of Lysocellin and Lasalocid A on the Distribution of Intramitochondrial Calcium

In coupled mitochondria, the fluorescence changes of chlorotetracycline are known to correlate with the active calcium accumulation by mitochondria and subsequent membrane binding[10]. The addition of 0.05 μM lysocellin or 0.1 μM lasalocid A (X–537A) caused a diminution in fluorescence, indicating calcium depletion from the mitochondrial inner membrane (Fig. 1). An apparent steady-state was established between energy-linked calcium accumulation and ionophore-induced depletion; this equilibrium declined and was diminished by the further additions of the ionophores, respiratory inhibitor rotenone or calcium chelator EDTA.

Effects of Lysocellin and Lasalocid A on Ca^{2+} and Mg^{2+} Movements by Mitochondria

Figure 2 shows the intramitochondrial Ca^{2+} and Mg^{2+} contents after treatment with various

Fig. 1. Effects of lysocellin and lasalocid A on the distribution of intramitochondrial calcium.

Mitochondria (1.8~2.0 mg) were incubated in the medium (3 ml) containing 10 mM MOPS-triethanolamine (pH 7.4), 10 mM acetate-triethanolamine (pH 7.4), 210 mM sucrose, 40 mM mannitol and 10 mM glutamate at 25°C.

Further additions at indicated points: the antibiotics at the concentration as described, 10 μM chlorotetracycline (CTC), 5 μM rotenone, 10 mM succinate, 3 mM ATP, 3 mM $MgCl_2$ and 2 mM ethylenediaminetetraacetic acid.

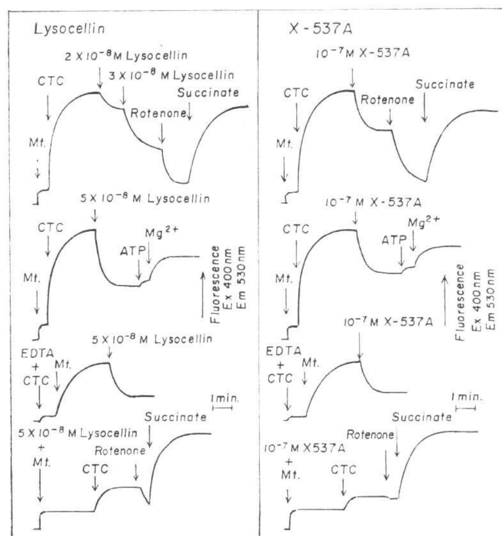
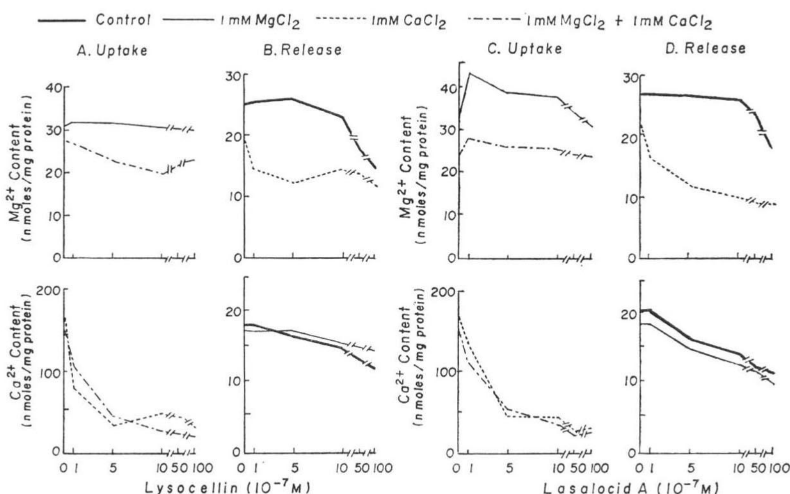


Fig. 2. Effects of lysocellin and lasalocid A on the intramitochondrial contents of Ca^{2+} and Mg^{2+}.
Mitochondria (3～4 mg protein) were incubated in the normal medium in the same way as described in Fig. 1 (final volume of 5 ml) with various concentrations of the antibiotics for 5 minutes at 27°C. For experimental details, see "Materials and Methods". Further additions were made: control, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $MgCl_2$ plus 1 mM $CaCl_2$.



concentrations of the antibiotics. At low concentrations of 0.5 μM, the additions of the compounds had no significant effect on Ca^{2+} and Mg^{2+} contents in mitochondria (Fig. 2–B, D). However, further increasing the concentration of the antibiotics to 10 μM induced a rapid efflux of Ca^{2+} and Mg^{2+} from mitochondria.

When mitochondria were incubated in a medium containing Ca^{2+} and oxidizable substrate, energy-linked Ca^{2+} uptake and Mg^{2+} release by mitochondria occurred. The uptake of calcium was inhibited by low levels of both compounds (0.5 μM) in the presence or absence of Mg^{2+} in the medium (Fig. 2–A,C). On the other hand, Mg^{2+} release induced by Ca^{2+} was augmented by low levels of the ionophores (0.1 μM), indicating a synergism between Ca^{2+} and ionophores, which was reversed by Mg^{2+} addition to the medium (Fig. 2–A, B, C, D).

Effect of Lysocellin on Alkali Metal Cation Transport and Light-scattering Changes Induced by Valinomycin or Monazomycin in Mitochondria

Valinomycin and monazomycin are known to stimulate mitochondrial uptake of alkali metal cations accompanied by oscillatory mitochondrial swelling[12,13]. When potassium uptake was induced by valinomycin in the presence of ATP, the addition of lysocellin (0.2 μM) caused rapid release of K^+ and reversed the mitochondrial swelling (Fig. 3–A). When uptake of alkali metal cations was stimulated by monazomycin in the presence of ATP, lysocellin produced contraction of mitochondria preloaded with K^+, Rb^+ or Na^+, but not with Li^+ or Cs^+ (Fig. 3–B).

Figure 4 shows the effect of lysocellin and its chemically modified derivatives on glutamate-supported swelling associated with K^+ uptake. Lysocellin was very potent in reversing swelling; even at 0.02 μM it produced contraction in mitochondria (Fig. 4–A). Chemical modification of terminal carboxylic acid or the C_{21} hydroxyl group significantly reduced the activity of the parent compound by one to two orders of magnitude (Fig. 4–B). On the other hand, lysocellin hexol (a derivative obtained by reduction

Fig. 3. Effect of lysocellin on alkali metal cation transport and light-scattering changes induced by valinomycin or monazomycin.
(A) A downward deflection of the light-scattering trace (L.S.) is associated with swelling of mitochondria. An upward deflection in the K^+ trace represents a decrease of its concentration in the medium or uptake of K^+ by mitochondria. The reaction system contained 20 mM triethanolaminechloride (pH 7.3), 10 mM acetate-triethanolamine (pH 7.3), 5 mM $MgCl_2$, 5 mM KCl, 5 mM Tris-ATP, 180 mM sucrose and 3 to 4 mg of mitochondrial protein. Final volume, 5 ml; temperature, 27°C. Antibiotic additions at indicated points: 10^{-7}M valinomycin, 0.2 μM lysocellin.
(B) The reaction system contained 20 mM Tris-acetate (pH 7.3), 5 mM $MgCl_2$, 30 mM alkali metal cation (Cl salt), 3 mM Tris-ATP, 150 mM sucrose and 0.2~0.3 mg of mitochondrial protein. Final volume, 3 ml: temperature, 27°C. Monazomycin and lysocellin were added at a concentration of 0.1 μM and 0.2 μM, respectively.

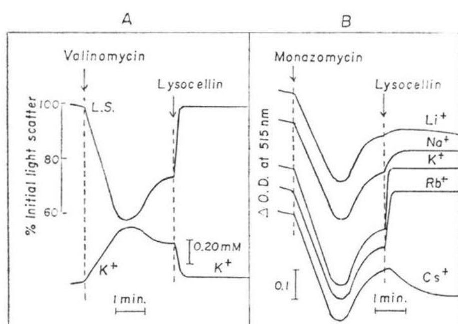
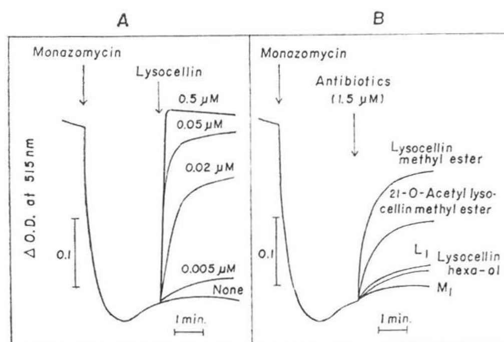


Fig. 4. Effects of lysocellin and its chemically modified compounds on mitochondrial swelling coupled to K^+ uptake induced by monazomycin.
The reaction system was the same as that described in Fig. 3-B except that glutamate (10 mM) was used as energy source instead of ATP. The antibiotics were added where indicated.



of both carbonyl functions at C_{11} and terminal carboxylic acid), the compound L_1 having a six-membered ring and a degradative compound M_1 exhibited little or no effect on mitochondrial swelling (Fig. 4–B).

Effect of Lysocellin on Substrate Oxidation in Mitochondria

At low concentrations of lysocellin, the antibiotic had no appreciable effect on respiration. However, 10 μM lysocellin stimulated state 4 respiration, and this stimulation was pronounced in the oxidation of succinate or β-hydroxybutyrate (Table 1). On the other hand, the antibiotic at 10 μM inhibited state 3 respiration or dinitrophenol-stimulated respiration, as shown in Table 1.

Effect on Mitochondrial Adenosine Triphosphatase

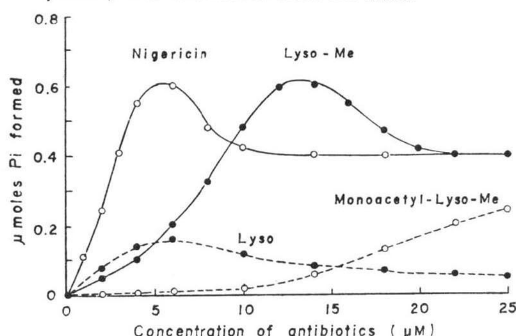
Table 1. Effect of lysocellin on substrate oxidation in mitochondria

Substrate	Stimulation (%)	Inhibition (%)	
	State 4 respiration	State 3 respiration	DNP-stimulated respiration
Glutamate	56	82	73
α-Ketoglutarate	50	81	69
Malate+Pyruvate	23	92	33
β-Hydroxybutyrate	392	25	23
Succinate	100	39	17

The reaction mixture contained 10 mM Tris-chloride, pH 7.4; 10 mM Tris-PO_4, pH 7.4, 20 mM KCl, 5 mM $MgCl_2$, 160 mM sucrose and 2~3 mg of mitochondrial protein. Final volume, 3 ml; temperature, 25°C. Respiration was previously stimulated by the addition of ADP (1 mM) or 2,4-dinitrophenol (20 μM), then lysocellin was added at a concentration of 10 μM.

The effects of lysocellin and its congeners are shown in Fig. 5. Lysocellin methyl ester (13 μM) strongly stimulated ATPase activity, whereas the parent compound and monoacetyl lysocellin methyl ester had little effect on the enzyme activity. This stimulation by lysocellin methyl ester depended on the presence of K^+ in the medium; less stimulation was observed in Na^+ or Rb^+ media but not in Li^+ or Cs^+ media. Nigericin is a unique ionophore because it causes K^+-dependent stimulation of mitochondrial ATPase (Ref. 14 or Fig. 5 in this paper). In our observations (data not shown), the K^+ specificity for ATPase stimulating activity of nigericin appeared more critical than that exhibited by lysocellin methyl ester.

Fig. 5. Effects of various ionophores on mitochondrial adenosine triphosphatase.
The reaction system contained 10 mM triethanolaminechloride (pH 7.4), 10 mM acetate-triethanolamine (pH 7.4), 40 mM KCl, 3 mM Tris-ATP, 100 mM sucrose and 0.2~0.3 mg of mitochondrial protein in a final volume of 1 ml. Incubation was conducted for 5 minutes at 30°C. The amount of inorganic phosphate hydrolyzed from ATP by control mitochondria during incubation (about 0.1 μmole) was subtracted from the data.



Discussion

It is apparent that lysocellin exhibits different effects on mitochondria depending upon the concentrations used. At low levels, it altered the fluorescence of chlorotetracycline, apparently as a result of inhibition of active calcium accumulation and depletion of membrane-bound calcium (Fig. 1). An apparent steady state was established between energy-linked Ca^{2+} accumulation and Ca^{2+} depletion induced by the ionophore. Under these experimental conditions, no appreciable change in mitochondrial Ca^{2+} and Mg^{2+} contents was detected by atomic absorption analysis (Fig. 2). However, low levels of the ionophore caused a strong inhibition of exogenously added calcium uptake and simultaneously released Mg^{2+} in a synergistic manner with Ca^{2+} (Fig. 2). These differential effects of lysocellin on Ca^{2+} and Mg^{2+} movements by mitochondria may be accounted for by the ability of mitochondria actively to accumulate Ca^{2+} and not Mg^{2+}. There was no fundamental difference in effect on cation movement between lysocellin and lasalocid A as shown in Figs. 1 and 2, in agreement with the fact that these ionophores display similar cation selectivity spectra in complexation with cations[3,8].

High levels of lysocellin produced changes of mitochondrial functions, such as release of state 4 respiration, stimulation of ATPase activity and inhibition of ADP- or DNP-stimulated respiration (Table 1 and Fig. 5). These uncoupling and inhibitory effects of the ionophore may be accounted for by the increased cycle of calcium influx and efflux and the loss of intramitochondrial Mg^{2+} and K^+. LIN and KUN[16] have reported a similar effect of lasalocid A on mitochondria and have proposed that it inhibits oxidative phosphorylation by irreversibly binding with a small amount of membrane-associated Mg^{2+}, which is considered to be part of the energy transducing catalytic system.

The data obtained by the use of analogues of lysocellin suggest the important role of terminal carboxylic acid and the C_{21} hydroxyl group in the activity of the ionophore (Figs. 4 and 5). This fact is consistent with our previous findings that the cation-complexing property of lysocellin decreased by chemical modification of these functions[3], indicating a good correlation between the effects on mitochondrial cation movements and the ability to form lipid-soluble cation-complexes. The finding that lysocellin methyl ester resembles nigericin-stimulated potassium-dependent ATPase activity in mitochondria indicates the complexity of mechanism of action of the compound.

In studies with the ionophore A23187, REED *et al.*[17] and PFEIFFER *et al.*[15,18] have reported the ionophore-induced depletion of intramitochondrial Ca^{2+} and Mg^{2+} and secondary movements of free phosphate and succinate and have proposed the mechanism of coupling between the transport processes

of cations and anions and the energy-producing metabolic reactions. Using the ionophores A23187, lasalocid A, nigericin and valinomycin, ROTTENBERG and SCARPA[19] studied in detail the relationship between active calcium accumulation and membrane potential in mitochondria. The effects of the ionophores on mitochondria are certainly involved in the cellular mechanism because mitochondria are considered to be the important organelles controlling cytosolic calcium levels[5,6,15]. Since the extent of cation selectivity of the ionophores in the biological system is not ascertained, it is necessary to discriminate between the primary and the secondary effects of the compounds in interpreting physiological data. PRESSMAN[8] has reported the intropic effects of monovalent cation selective ionophores as well as lasalocid A and proposed that these activities are probably due to their sodium transport activity, which indirectly regulates cytosolic calcium levels and consequently induces exocytotic secretion of biogenic amines. We showed with rabbit blood platelets[4] that a monovalent cation ionophore etheromycin (CP-38,295), as well as lysocellin and lasalocid A, caused platelet aggregation by inducing serotonin secretion and mobilization of intracellular calcium. Preliminary results with other monovalent cation ionophores indicated that the monensins, A-204, septamycin, Ro 21-6150, dianemycin, X-206, salinomycin and nigericin all induced blood platelet aggregation. These findings, together with other preliminary data showing that the low levels of ionophores produced redistribution of calcium associated with mitochondrial inner membrane, indicate the complexity of their mode of action. Further study is required to describe the mechanism of action of the ionophores.

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