STUDIES ON THE IONOPHOROUS ANTIBIOTICS. XVI[1]

THE IONOPHORE-MEDIATED CALCIUM TRANSPORT AND CONCOMITANT OSMOTIC SWELLING OF MITOCHONDRIA

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The effects of various carboxylic ionophores on divalent metal cation translocation in mitochondria have been investigated. High levels of divalent cation ionophores lysocellin and lasalocid A (10~50 μM) produced mitochondrial osmotic swelling in Ca^{2+} or Mg^{2+} medium, which was associated with an increase of cation influx. The extent of swelling was a function of both the ionophore and cation concentrations in the medium. This effect was larger in mitochondria de-energized by treatment with antimycin A and oligomycin than in respiring mitochondria. On the other hand, the monovalent cation ionophores carriomycin and etheromycin at concentrations of 50~100 μM also induced mitochondrial swelling in Ca^{2+} medium but were ineffective in Mg^{2+} medium. Addition of ruthenium red reversed divalent cation ionophore-induced swelling and released Ca^{2+} from preloaded mitochondria. In contrast, ruthenium red increased monovalent cation ionophore-induced swelling. In a divalent cation-free medium, lysocellin and lasalocid A caused depletion of membrane-bound Ca^{2+} and released endogenous Ca^{2+} and Mg^{2+} from mitochondria, while carriomycin and etheromycin exerted only a limited effect. These results indicate that the divalent cation ionophores affect divalent cation distribution in mitochondria by increasing both influx and efflux of the cations through the inner membrane.

Carboxylic ionophores catalyze an electroneutral exchange of cations for H^+ across biological membranes and thereby selectively alter membrane permeability to cations.[2,3] These compounds have served as useful tools for studying the molecular mechanism of both cation transport systems and cation-dependent biological processes.[4~6] In mitochondria, these antibiotics cause depletion of intramitochondrial alkali and alkaline earth metal cations and induce a secondary release of anions, thereby inhibiting mitochondrial energy transducing reactions.[7~9] These compounds, therefore, are considered to mimic naturally occurring cation/H^+ antiport in the mitochondrial inner membrane and cause a net extrusion of cations.[2,10] On the other hand, it is known that the neutral ionophores such as valinomycin and synthetic ligands, which form positively-charged complexes with cations, promote the electrophoretic influx of cations by mitochondria in response to electrical potential gradient on the inner membrane.[11,12]

We have previously reported[13] that a monovalent cation ionophore etheromycin (CP-38295 or T-40517) and the calcium ionophores lasalocid A and lysocellin mobilized the intracellular calcium of blood platelets, thus inducing the secretion reaction and aggregation. Since etheromycin is thought to exert this activity through increasing alkali metal cation movement, these findings led us to consider a probable regulatory role of monovalent cations on calcium metabolism as proposed by LOWE *et al.*[14]

In the present study, by the use of ionophores with various cation selectivity profiles, we show

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some experimental evidence suggesting that monovalent cation ionophores are able to influence divalent metal cation translocation in mitochondria, although the activity is small and limited as compared with that of divalent cation ionophores.

Materials and Methods

Chemicals. The antibiotics used in this report were obtained from the following sources; lysocellin, carriomycin, etheromycin, antimycin A and oligomycin were the stock samples in our laboratory. Lasalocid A and A 23187 were generous gifts from Dr. J. G. WHITNEY of Eli Lilly and Co., Indianapolis, Ind., U.S.A. Chlorotetracycline was purchased from Calbiochem., San Diego, Calif., U.S.A. and ^{45}Ca with high specificity from New England Nuclear, Boston, Mass., U.S.A., respectively.

Mitochondria. Mitochondria were prepared from male rat livers as described by JOHNSON and LARDY.[15] The homogenizing medium contained 225 mM mannitol, 75 mM sucrose, 2 mM Tris-chloride (pH 7.4) and 1 mM EDTA. The washing was carried out in an EDTA free medium.

Measurement of Ca^{2+} and Mg^{2+} translocation in mitochondria. For determination of ^{45}Ca uptake, aliquots of mitochondrial suspension were filtered through Millipore filters and washed twice with 210 mM mannitol - 70 mM sucrose solution containing 20 mM acetate-triethanolamine (pH 7.4). Then, the radioactivity retained by the filters was determined. The intramitochondrial contents of Ca^{2+} and Mg^{2+} were determined by atomic absorption analysis or by the fluorescence probe chlorotetracycline technique as described previously.[6] Mitochondrial osmotic swelling was monitored spectrophotometrically by measuring the changes of absorbance at 520 nm.

Results

The Ionophore-induced Mitochondrial Swelling

It is well known that in the presence of permeant anions, there is a net uptake of cations by respiring mitochondria followed by an influx of water and oscillatory swelling.[17] Addition of the divalent cation ionophores lysocellin, lasalocid A or A 23187 caused rapid swelling of mitochondria suspended in Ca^{2+} medium (Fig. 1). Lysocellin induced the swelling in Mg^{2+} medium, whereas lasalocid A and A 23187 were less effective. The extent of the ionophore-induced swelling was dependent on both the ionophore and cation concentrations in the medium. The presence of oxidizable substrates did not influence the antibiotic activity. On the other hand, high levels of monovalent cation ionophores carriomycin and etheromycin produced little swelling in Ca^{2+} medium and no swelling in Mg^{2+} medium (Fig. 1). When mitochondria were de-energized by treatment with the respiratory-chain inhibitors antimycin A and oligomycin, a moderate swelling was observed when Ca^{2+} or Mg^{2+} were added (Fig. 2). Subsequent addition of divalent cation ionophores promoted mitochondrial swelling in both Ca^{2+} and Mg^{2+} media (Fig. 2). The monovalent cation ionophores also produced an appreciable swelling in Ca^{2+} medium but did not in Mg^{2+} medium. The addition of ruthenium red, a potent inhibitor of energy-linked calcium transport,[18] reversed divalent cation ionophore-induced swelling and contraction of mitochondria (Figs. 1 and 2). A similar contraction of mitochondria was also observed by another calcium transport inhibitor $LaCl_3$. In contrast, ruthenium red increased the monovalent cation ionophore-induced swelling of de-energized mitochondria in both Ca^{2+} and Mg^{2+} media (Fig. 2).

The Ionophore-induced ^{45}Ca Uptake by Mitochondria

As shown in Fig. 3, lysocellin and lasalocid A promoted the influx of externally added ^{45}Ca into mitochondria treated with antimycin A and oligomycin. Subsequent addition of ruthenium red caused release of accumulated ^{45}Ca from mitochondria (Fig. 3). Carriomycin and etheromycin did not

Fig. 1. Induction of mitochondrial swelling by the ionophores in Ca^{2+} or Mg^{2+} medium.

Mitochondria (0.05~0.1 mg) were incubated in the medium (total volume of 3 ml) containing 10 mM acetate-triethanolamine (pH 7.4), 10 mM MOPS-triethanolamine (pH 7.4), 210 mM mannitol and 70 mM sucrose at 25°C. Further additions at indicated points: 1 mM Ca^{2+}, 1 mM Mg^{2+}, 16 μM lysocellin, 50 μM lasalocid A, 8 μM A 23187, 100 μM carriomycin, 100 μM etheromycin and 10 μM ruthenium red.

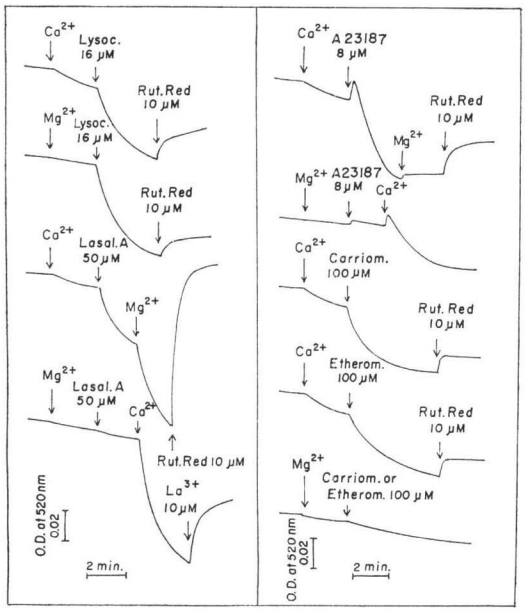
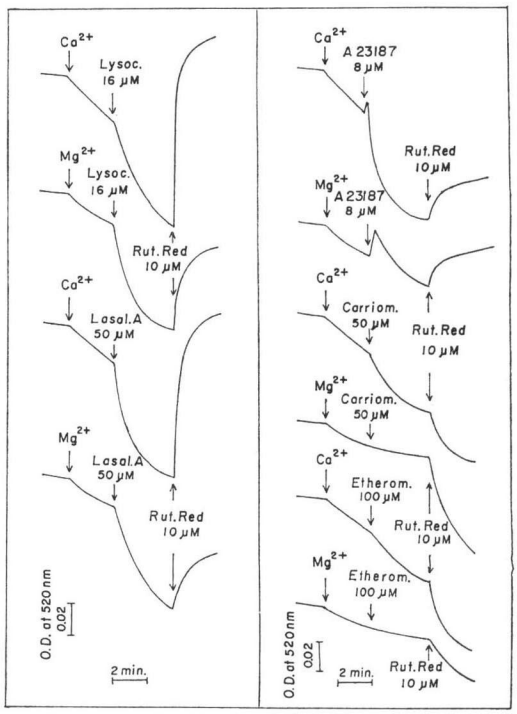


Fig. 2. Ionophore-induced swelling of mitochondria de-energized by treatment with antimycin A and oligomycin in Ca^{2+} or Mg^{2+} medium.

Mitochondria (0.05~0.1 mg) were incubated in the normal medium as described in Fig. 1 with further additions of 1.6 μg per ml of antimycin A and oligomycin. Additions indicated in the figure were the same as described in Fig. 1.



change ^{45}Ca uptake either in the presence or absence of ruthenium red (Fig. 3). It is uncertain whether conformational change of mitochondria induced by monovalent cation ionophores associates directly with the increased cation flux.

Effects of the Ionophores on the Distribution of Intramitochondrial Ca^{2+} and Mg^{2+}

The fluorescence probe with chlorotetracycline is a useful indicator of membrane-bound calcium and its fluorescence signal is altered by the active accumulation and depletion of calcium.[8,19] The addition of 0.05 μM lysocellin or 0.1 μM lasalocid A to respiring mitochondria decreased fluorescence, indicating depletion of membrane-bound calcium (Fig. 4). An apparent steady-state of calcium distribution was observed across the inner membrane responding to the

Fig. 3. Effects of the ionophores on the uptake of exogenously-added ^{45}Ca by mitochondria.

The reaction system was the same as described in Fig. 2 (final volume of 5 ml). Mitochondria (0.3~0.4 mg) were preincubated with 1.6 μg per ml of antimycin A and oligomycin and the reaction was started by the addition of 1 mM $^{45}CaCl_2$. Further additions were as follows: 16 μM lysocellin, 50 μM lasalocid A, 100 μM carriomycin, 100 μM etheromycin and 10 μM ruthenium red.

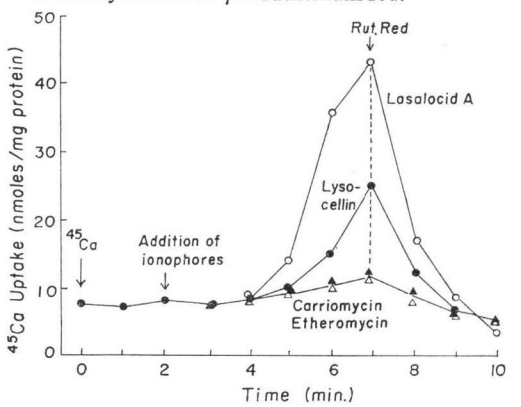


Fig. 4. Effects of various ionophores on the distribution of membrane-bound calcium of mitochondria.
The reaction system contained 10 mM acetate-triethanolamine, 10 mM MOPS-triethanolamine (pH 7.4), 210 mM mannitol, 70 mM sucrose, 10 mM glutamate and 1.8~2.2 mg of mitochondrial protein. Final volume, 3 ml; temperature 27°C. Further additions at indicated points: the antibiotics at a concentration described, in the figure, 10 μM chlorotetracycline (CTC), 5 μM rotenone, 10 mM succinate, 3 mM ATP, 3 mM $MgCl_2$ and 2 mM ethylenediaminetetraacetic acid.

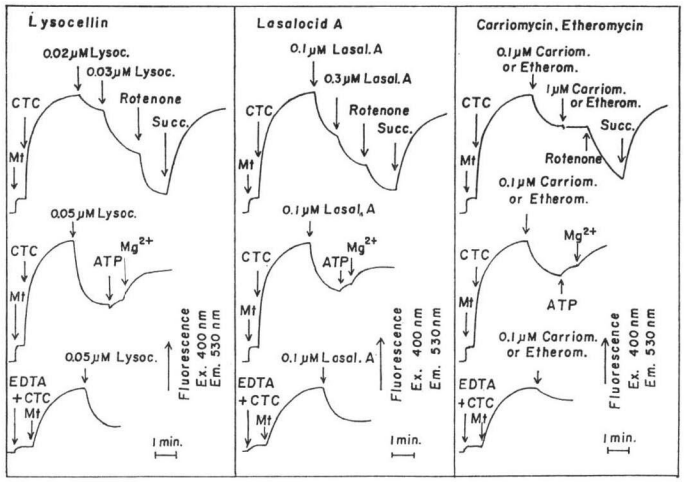
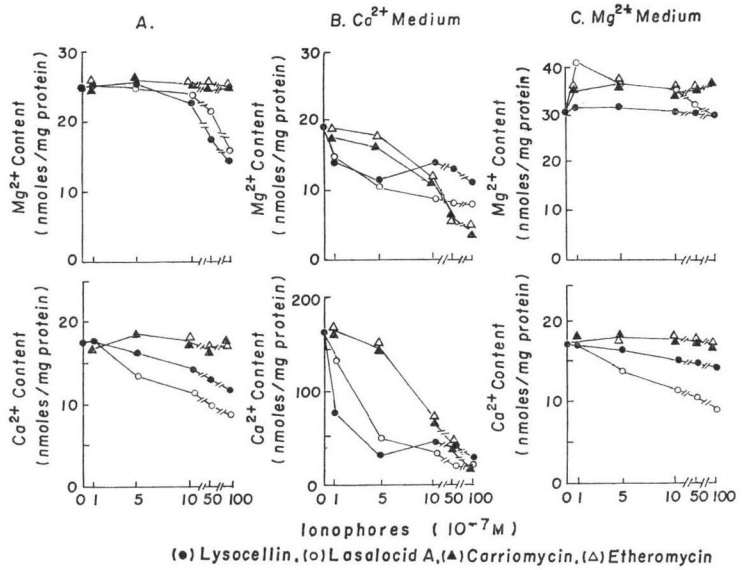


Fig. 5. Effects of various ionophores on the intramitochondrial contents of Ca^{2+} and Mg^{2+}.
Mitochondria (3~4 mg protein) were incubated in the normal medium as described in Fig. 4 (A) and the medium which contained in addition 1 mM $CaCl_2$ (B) or 1 mM $MgCl_2$ (C). Final volume, 5 ml; temperature, 27°C. After 5-minute reaction with various concentrations of the antibiotics, mitochondrial contents of Ca^{2+} and Mg^{2+} were determined by atomic absorption analysis. Antibiotic additions: lysocellin, lasalocid A, carriomycin and etheromycin.



antibiotic concentrations in the medium. This equilibrium was lowered by further addition of the ionophores, the respiratory inhibitor rotenone or the calcium chelator EDTA. On the other hand, carriomycin and etheromycin at 0.1 μM produced a small fluorescence decrease (Fig. 4). No further

change in fluorescence was observed even if a high concentration of antibiotics was added. The addition of Mg^{2+} plus ATP partially reversed the ionophore-induced fluorescence decrease, indicating an energy-dependent reaccumulation of Mg^{2+}.

Ca^{2+} and Mg^{2+} content of mitochondria treated with various antibiotic concentrations was measured by atomic absorption analysis (Fig. 5). Low levels of the antibiotics did not change intramitochondrial cation content, whereas a high concentration of lysocellin or lasalocid A released endogenous Ca^{2+} and Mg^{2+} (Fig. 5A). In a Ca^{2+} medium, lysocellin or lasalocid A at 0.5 μM and carriomycin or etheromycin at 5 μM partially inhibited the energy-linked calcium uptake as shown in Fig. 5B. Under these conditions, the antibiotics caused Mg^{2+} depletion from mitochondria in a synergistic manner with calcium. This synergistic effect was prevented by the addition of Mg^{2+} to the medium (Fig. 5C).

Discussion

It is well established that the ionophore antibiotics affect mitochondrial energy-transducing processes which are associated with cation transport systems by perturbing cation translocation in the inner membrane.[2~4,7~9] Our present study showed that the divalent cation ionophores lysocellin and lasalocid A depleted membrane-associated calcium at a low concentration and produced simultaneous induction of calcium influx and efflux through the inner membrane at a high concentration. DUSZYNSKI and WOJTCZAK[20] have recently reported a similar effect of A 23187 on the swelling of mitochondria suspended in isotonic KNO_3 and $Mg(NO_3)_2$. It is apparent that these activities of the antibiotics are due to their function as a mobile carrier of cations.

The monovalent cation ionophores carriomycin and etheromycin in common with divalent were able to influence Ca^{2+} and Mg^{2+} translocation in mitochondria, although the activity was quite small and limited. There was a significant difference in the effects of mono- and divalent cation ionophores on mitochondrial swelling in the presence of ruthenium red (Fig. 2). The addition of ruthenium red reversed divalent cation ionophore-induced swelling but, in contrast, increased monovalent cation ionophore-induced swelling. It is known that ruthenium red inhibits both energy-linked influx and efflux of Ca^{2+} and the binding of it at the divalent cation carrier site depends on a negative-inside electrical potential of membrane.[18] In the meantime, ROTTENBERG and SCARPA[4] have reported that the monovalent cation ionophores nigericin and valinomycin change membrane potential and ΔpH on mitochondrial membrane, thereby inducing influx or efflux of calcium. It therefore is conceivable that transmembrane electric potential and ΔpH must take part in calcium transport activity of both the ionophores and ruthenium red.

It has been suggested[14] that an increase of intracellular sodium produces an increase of cytosolic calcium levels by liberating it from the intracellular pools. PRESSMAN[2] has reported that the monovalent cation ionophores as well as lasalocid A exhibited inotropic activity in intact dogs and proposed that this effect may be due to their sodium transport activity, which indirectly increases cytosolic calcium and thereby induces exocytotic release of biogenic amines. In addition, KNAPP *et al.*[21] have recently reported that nigericin and monensin A as well as A 23187 and lasalocid A produced calcium-dependent stimulation of prostaglandin synthesis in renal medulla. These observations together with our findings[13] of platelet aggregation effect suggest the possibility that these antibiotics are able to trigger calcium-dependent biological processes through increasing alkali metal cation movement, especially of Na^+. Since mitochondria are considered to play an important role in regulation of cytosolic calcium levels, the effects of mono- and divalent cation ionophores on mitochondrial divalent cation distribution described in this report, are considered to be involved in their action at cellular levels.

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