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Alterations in mitochondrial Ca^{2+} flux by the antibiotic X-537A (lasalocid-A)

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A previous communication (Pereira da Silva, L., Bernardes, C.F. and Vercesi, A.E. (1984) Biochem. Biophys. Res. Commun. 124, 80–86) presented evidence that lasalocid-A, at concentrations far below those required to act as a Ca^{2+} ionophore, significantly inhibits Ca^{2+} efflux from liver mitochondria. In the present work we have studied the mechanism of this inhibition in liver and heart mitochondria. It was observed that lasalocid-A (25–250 nM), like nigericin, promotes the electroneutral exchange of K^+ for H^+ across the inner mitochondrial membrane and as a consequence can cause significant alterations in ΔpH and $\Delta\psi$. An indirect effect of these changes that might lead to inhibition of mitochondrial Ca^{2+} release was ruled out by experiments showing that the three observed patterns of lasalocid-A effect depend on the size of the mitochondrial Ca^{2+} load. At low Ca^{2+} loads (5–70 nmol Ca^{2+}/mg protein), under experimental conditions in which Ca^{2+} release is supposed to be mediated by a $Ca^{2+}/2H^+$ antiporter, the kinetic data indicate that lasalocid-A inhibits the efflux of the cation by a competitive mechanism. The $Ca^{2+}/2Na^+$ antiporter, the dominant pathway for Ca^{2+} efflux from heart mitochondria, is not affected by lasalocid-A. At intermediate Ca^{2+} loads (70–110 nmol Ca^{2+}/mg protein), lasalocid-A slightly stimulates Ca^{2+} release. This effect appears to be due to an increase in membrane permeability caused by the displacement of a pool of membrane bound Mg^{2+} possibly involved in the maintenance of membrane structure. Finally, at high Ca^{2+} loads (110–140 nmol Ca^{2+}/mg protein) lasalocid-A enhances Ca^{2+} retention by liver mitochondria even in the presence of Ca^{2+}-releasing agents such as phosphate and oxidants of the mitochondrial pyridine nucleotides. The maintenance of a high membrane potential under these conditions may indicate that lasalocid-A is a potent inhibitor of the Ca^{2+}-induced membrane permeabilization. Nigericin, whose chemical structure resembles that of lasalocid-A, caused similar results.

Introduction

Calcium distribution across the inner mitochondrial membrane under steady-state conditions is kinetically determined by a continuous cyclic movement of the cat ion through independent pathways for influx and efflux (cf. Refs. 1–4). The influx pathway is well characterized as an electrophoretic uniporter [5,6], while the efflux of the cation is mediated by an Na^+-dependent or an Na^+-independent pathway [7]. The dominant pathway for Ca^{2+} efflux in mitochondria from non-excitable tissues is independent of Na^+ [8] while the Na^+-dependent pathway predominates in mitochondria from excitable tissues [7].

The Na^+-dependent release pathway appears to be an electroneutral $2Na^+/Ca^{2+}$ exchanger [9]. Although an electroneutral $Ca^{2+}/2H^+$ antiporter has been proposed for the Na^+-independent pathway, a wide variation in Ca^{2+}/H^+ stoichiometry is observed if measurements are carried out over a range of medium pH [10]. Moreover, other observations indicate that the efflux of Ca^{2+} does not increase with increasing pH gradient across the membrane, and no Ca^{2+}-Ca^{2+} exchange has been demonstrated to occur through this pathway [10]. These characteristics of the Na^+-independent pathway argue against the mechanism being a passive $Ca^{2+}/2H^+$ exchange [4].

Abbreviations: $\Delta\tilde{\mu}H^+$, electrochemical proton gradient; $\Delta\psi$, transmembrane electrical potential; BSA, bovine serum albumin; NEM, *N*-ethylmaleimide; TPP^+, tetraphenylphosphonium; RR, Ruthenium red; RLM, rat liver mitochondria; RHM, rat heart mitochondria: LA, lasalocid-A; Nig, nigericin; Mal, malonate; *t*-butOOH, *tert*-butylhydroperoxide.

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An NAD(P)$^+$-stimulated Ca^{2+} release from liver, heart and tumor mitochondria was reported about 10 years ago [11]. Despite much study (for reviews see Refs. 4,12,13), the molecular mechanism responsible for this Ca^{2+} release remains poorly understood and controversial. The finding that a decrease in membrane potential parallels mitochondrial swelling during Ca^{2+} efflux even in mitochondria containing low Ca^{2+} concentrations (10 nmol Ca^{2+}/mg) and protected by physiological concentrations of ATP and Mg^{2+} [14] does not support the original idea [11] that this could represent a regulatory mechanism. Rather, it has been proposed [14,15] that this process could be a factor in the mechanism of cell toxicity caused by disruption of intracellular Ca^{2+} homeostasis associated to oxidative stress [16].

Although results from Crompton's laboratory have characterized this Ca^{2+} efflux pathway as a specific protein-mediated process inhibited by cyclosporin A [17,18], recent results from our laboratory strongly indicate that this Ca^{2+}-induced hole result from non-specific membrane protein disulfide linkages [19,20]. We have recently reported [21] that the antibiotic X-537A (lasalocid-A), at concentrations far below those required for activity as a Ca^{2+} ionophore inhibits Ca^{2+} release from rat liver mitochondria. In the present work we have studied the mechanism of this inhibition. The results indicate that lasalocid-A inhibits both the carrier-mediated Ca^{2+} release and the opening of the Ca^{2+}-induced hole.

Material and Methods

Isolation of mitochondria. Liver mitochondria were isolated by the method of Schneider and Hogeboom [22] and heart mitochondria by the method of Vercesi et al. [23] from overnight fasted male Wistar rats weighing approx. 250 g. The mitochondrial pellets were resuspended in a medium containing 250 mM sucrose and 3 mM Hepes (pH 7.2), and the protein content was determined by the biuret method modified by the addition of cholate.

Standard incubation procedure. The experiments were carried out at 30 °C in a basic medium containing 125 mM sucrose, 65 mM KCl, 3.0 mM Hepes buffer (pH 7.2), 1.0 mM $MgCl_2$, 4.0 μM rotenone, 0.1 mg/ml bovine serum albumin and 2.0 mM succinate. Other modifications of the basic medium are described in the figure legends.

Determination of Ca^{2+} movements. Changes in Ca^{2+} concentration in the suspending medium were followed using a Ca^{2+}-selective electrode (Radiometer, F2112 Calcium Selectrode). Signals from the Ca^{2+} electrode were fed into an amplifier and then into a dual-channel strip-chart recorder (Linear model 1202). The response of the Ca^{2+} electrode was calibrated in each system by addition of internal standards to the medium. Ca^{2+} release was also determined by atomic absorption spectrophotometry in a Perkin Elmer 303 atomic absorption spectrophotometer. Samples of 2.5 ml were taken from the incubation medium following Ruthenium red addition and centrifuged at 10000 × g. Calcium was determined in the supernatant according to Golchman and Givelbert [24].

Determination of mitochondrial swelling. Mitochondrial swelling was estimated from the decrease in the absorbance at 540 nm measured in a Beckman spectrophotometer.

Measurements of mitochondrial transmembrane electrical potential. Mitochondria were incubated in the reaction medium containing 3 μM tetraphenylphosphonium (TPP^+). The concentration of TPP^+ in the extramitochondrial medium was continuously monitored with a TPP^+-selective electrode prepared in our laboratory according to Kamo et al. [25]. The mitochondrial membrane potential was then calculated assuming that the TPP^+ distribution between mitochondria and medium follows the Nernst equation [26].

Determination of membrane-bound Mg^{2+}. Mitochondria (0.5 mg · ml^{-1}) were incubated in a medium containing 125 mM sucrose, 65 mM KCl, 3 mM Hepes buffer (pH 7.2), 2 mM succinate, 0.2 mM P_i, 4 μM rotenone and 0.25 mM EGTA. 10 μM chlorotetracycline was added and the fluorescence was measured in an Aminco Bowman spectrofluorimeter at 365 → 530 nm. According to Caswell and Hutchison [27] the fluorescence intensity is associated to divalent cations, mainly Mg^{2+}, bound to the inner surface of the inner membrane. Addition of lasalocid-A (250 pmol · mg^{-1}) decreased the fluorescence, which could be restored upon addition of 4 mM Mg^{2+} and 3 mM ATP, in agreement with the results published by Lin and Kun [28].

Chemicals. Nigericin, Rotenone, ATP, *t*-butylhydroperoxide, Hepes, EGTA, oligomycin and NEM were obtained from Sigma Chemical Company, TPP^+ from Aldrich, and lasalocid-A from Hoffman-La Roche; all other reagents were commercial products of the highest available grade of purity.

Results

In a previous communication [21] we have shown that lasalocid-A, at concentrations far below those required to act as a Ca^{2+} ionophore, significantly inhibits Ca^{2+} efflux from liver mitochondria. Since mitochondrial Ca^{2+} efflux takes place through a carrier-mediated process (Na^+-dependent or Na^+-independent mechanism) or through nonspecific pathways induced by the accumulation of excess Ca^{2+}, it was important to clarify which of these mechanisms were sensitive to the antibiotic. With this purpose we have first studied the effect of lasalocid-A on the rates of Ca^{2+} release from liver

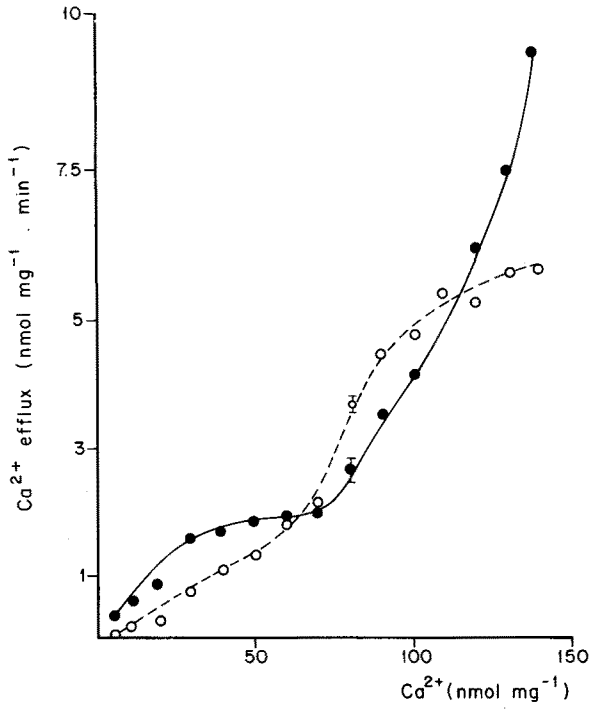

Fig. 1. Effect of lasalocid-A on the ruthenium red induced Ca^{2+} efflux from liver mitochondria under variable Ca^{2+} loads. Mitochondria (1 mg) were suspended in 1.0 ml of basic medium (see Materials and Methods) without Mg^{2+} and BSA. P_i (0.5 mM) and Ca^{2+} (5–140 nmol/mg protein) were added in the absence (———) or presence (— — —) of 250 pmol lasalocid-A. The rates of Ca^{2+} efflux were determined after the addition of 0.7 μM Ruthenium red using a Ca^{2+}-selective electrode.

mitochondria containing different Ca^{2+} loads (5 to 146 nmol Ca^{2+}/mg protein).

The rates of Ca^{2+} efflux were measured after addition of Ruthenium red to mitochondria that had taken up Ca^{2+} until a steady-state Ca^{2+} distribution had been attained. The solid line in Fig. 1 shows the rates of Ruthenium red-induced Ca^{2+} efflux in the absence of lasalocid-A. The rates of Ca^{2+} release increased with increasing the mitochondrial loads and the saturation (V_{max} of 2 nmol · min^{-1} · mg^{-1}) was attained at a Ca^{2+} load of about 25 nmol/mg. However, at concentrations higher than 70 nmol Ca^{2+}/mg protein the rates of Ca^{2+} release increased dramatically as Ca^{2+} load increased, reaching values of about 10 nmol Ca^{2+} · min^{-1} · mg^{-1} in mitochondria loaded with 140 nmol Ca^{2+}/mg protein. The dashed line shows a complex effect of lasalocid-A on the rates of Ca^{2+} release. The degree of inhibition observed at 5 nmol Ca^{2+}/mg protein (about 60%) decreased progressively to 0% at 60–70 nmol Ca^{2+}/mg protein. In the range between 70 and 110 nmol Ca^{2+}/mg protein, lasalocid-A significantly increased the rates of Ca^{2+} release and at concentrations higher than 110 nmol Ca^{2+}/mg protein, again a strong inhibition was observed. This figure is representative of three similar experiments performed with different mitochondrial preparations. Although the stimulation of Ca^{2+} release by lasalocid-A was always observed within a range of Ca^{2+} concentration, the limits of this range varied with different preparations. The higher stimulation (37%) of Ca^{2+} release by lasalocid-A in Fig. 1 occurred at a Ca^{2+} load of 80 nmol Ca^{2+}/mg protein and the rates of Ca^{2+} release at this Ca^{2+} concentration represents the average of four experiments.

Fig. 2 shows the effect of lasalocid-A on Ca^{2+} efflux when mitochondria were loaded with 5 to 150 nmol Ca^{2+}/mg protein in the presence of ATP and Mg^{2+}, which protect mitochondria from undergoing the Ca^{2+}-dependent increase in membrane permeability [2]. In this case the degree of inhibition by lasalocid-A decreased as the Ca^{2+} load increased but a residual inhibition (about 25%) was still observed at a Ca^{2+} load close to 100 nmol/mg protein. The dramatic increase in Ca^{2+} efflux, which was observed at about 70 nmolCa^{2+}/mg in the absence of lasalocid-A, ATP and Mg^{2+} (Fig. 1), was displaced to higher Ca^{2+} loads (about 120 nmol Ca^{2+}/mg) in the presence of ATP and Mg^{2+}. Under these conditions the stimulation of Ca^{2+} release caused by the antibiotic in the range of 70 to 110 nmol Ca^{2+}/mg was completely absent.

Under some conditions, lasalocid-A acts as a K^+/H^+ exchanger across biological membranes [29] and this exchange can alter the relative values of $\Delta\psi$ and ΔpH across the inner mitochondrial membrane. Since alterations in these parameters may affect the rates of Ca^{2+} release from mitochondria [2,30], we have studied the activity of lasalocid-A as a K^+/H^+ exchanger in the

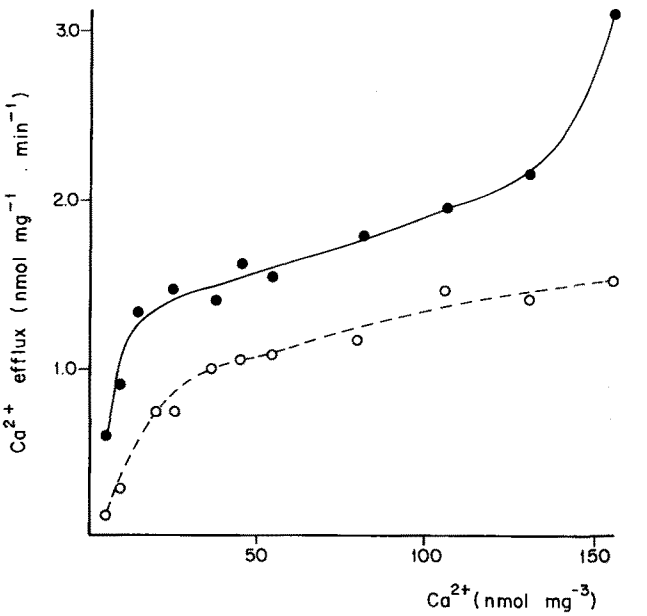

Fig. 2. Effect of lasalocid-A on Ruthenium-red-induced Ca^{2+} efflux from liver mitochondria under variable Ca^{2+} loads in the presence of ATP and Mg^{2+}. The experimental conditions were the same as in Fig. 1, except that 0.6 mM ATP, 0.6 mM MgCl2 and 0.5% BSA were also present.

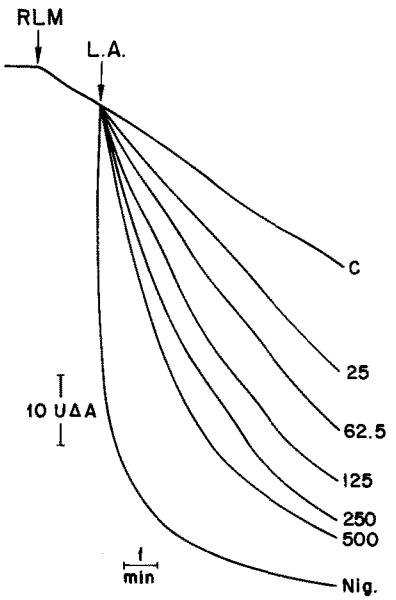

Fig. 3. Swelling induced by lasalocid-A in mitochondria suspended in potassium acetate medium. Lasalocid-A (25 to 500 pmol) or 0.5 μg nigericin were added where indicated to 0.5 mg mitochondrial protein incubated in 2.0 ml of 100 mM potassium acetate containing 1.0 μg/ml oligomycin and 5 μM rotenone. Trace c represents the experiment carried out in the absence of lasalocid-A.

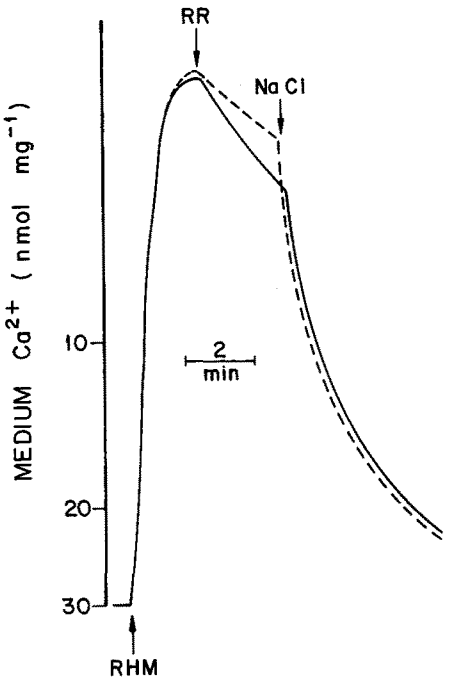

Fig. 5. Effect of lasalocid-A on sodium-independent and sodium-dependent Ca^{2+} efflux from heart mitochondria. Heart mitochondria (1 mg protein) were incubated in 1.0 ml of the same medium described in Fig. 3, without TPP^+, in the absence (———) or presence (— — —) of 250 pmol lasalocid-A. Ruthenium red (0.6 μM) and NaCl (5.0 mM) were added where indicated.

range of concentrations in which it inhibits Ca^{2+} release from mitochondria. Fig. 3 shows that, like nigericin, lasalocid-A caused swelling of deenergized liver mitochondria suspended in potassium acetate in a dose-dependent manner. The results in Fig. 3 suggest that the addition of lasalocid-A to energized mitochondria, under our conditions would cause an increase in $\Delta\psi$, due to the collapse of ΔpH, by promoting the exchange of internal K^+ for external H^+. In fact, Fig. 4 shows an increase of about 15 mV in the value of the membrane potential following the addition of lasalocid-

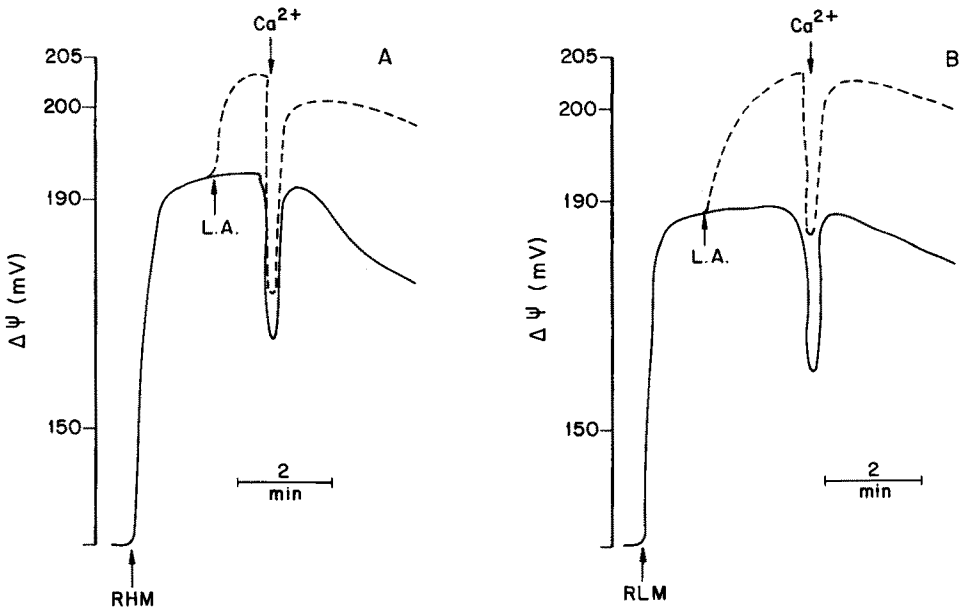

Fig. 4. Changes in transmembrane electrical potential induced by lasalocid-A in liver and heart mitochondria. Heart (A) or liver (B) mitochondria (1mg protein) were incubated in 1.0 ml of a medium containing 130 mM KCl, 3 mM Hepes, 5 mM succinate, 5 μM rotenone and 3 μM TPP^+. Lasalocid-A (250 pmol) (— — —) and 20 nmol Ca^{2+} were added where indicated. The experiments in the absence of lasalocid-A are represented by the continuous traces.

A to liver (A) or heart (B) mitochondria. This figure also shows that the changes in $\Delta\psi$ caused by Ca^{2+} addition were not significantly altered by the antibiotic; however, $\Delta\psi$ is better sustained by both types of mitochondrion in the presence of lasalocid-A.

The effect of lasalocid-A on Ca^{2+} release from heart mitochondria is shown in Fig. 5. It can be seen that the rate of Ruthenium red-induced Ca^{2+} release was slower in the presence of lasalocid-A (dashed line). The subsequent addition of NaCl to this preparation greatly stimulated Ca^{2+} release; however, the rates of this

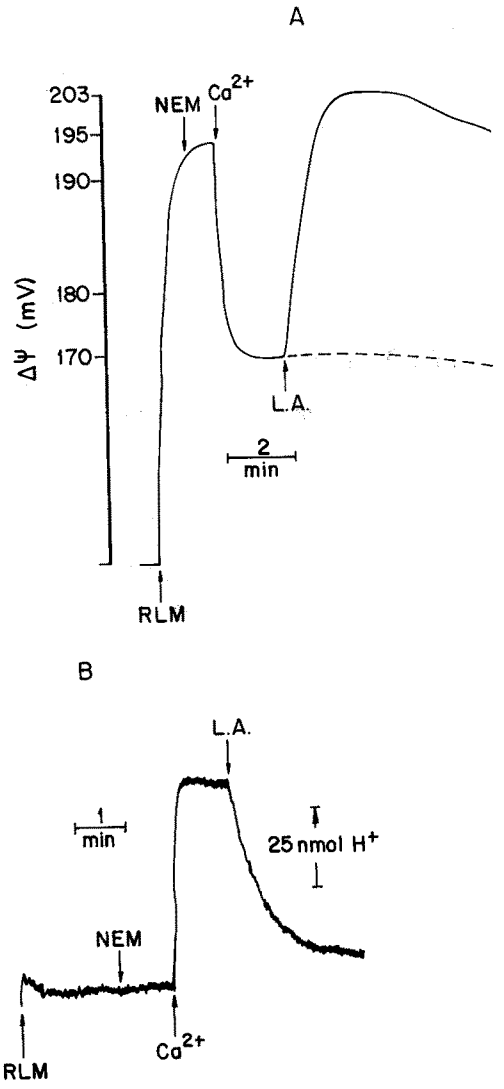

Fig. 6. Changes in $\Delta\psi$, ΔpH and medium pH induced by lasalocid-A addition to liver mitochondria loaded with Ca^{2+} in the presence of *N*-ethylmaleimide. Liver mitochondria (1 mg protein) was added to 1.0 ml of basic medium plus 3 μM TPP^+. NEM (60 μM), Ca^{2+} (20 nmol) and lasalocid-A (100 pmol) were added where indicated. The values of ΔpH were estimated applying the equation $\Delta\tilde{\mu}_{H^+} = \Delta\psi - 60\Delta$pH, considering a maximum value of 203 mV for $\Delta\tilde{\mu}_{H^+}$, which is equal to $\Delta\psi$, when ΔpH is zero. This condition was established by the additions of lasalocid-A or nigericin concentrations which caused the maximal increase in $\Delta\psi$. Alterations in $\Delta\psi$ (A) and medium pH (B) were followed with specific electrodes.

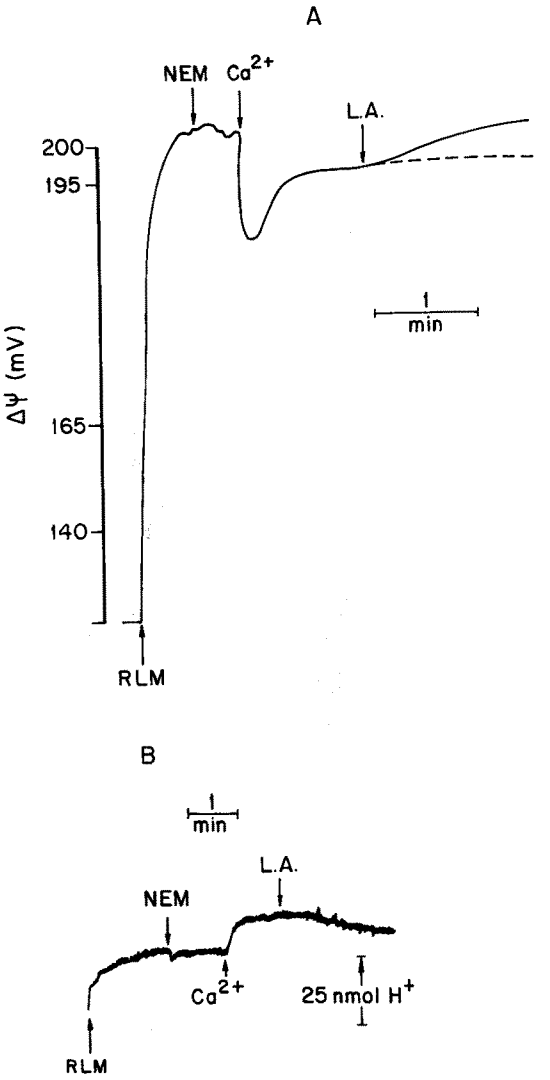

Fig. 7. Effect of lasalocid-A on $\Delta\psi$ and medium pH in the presence of potassium acetate. Liver mitochondria (1 mg protein) were incubated in 1.0 ml of the basic medium containing 30 mM KAc and 3 μM TPP^+. NEM (60 μM), Ca^{2+} (20 nmol) and lasalocid-A (100 pmol) were added where indicated (———). The dashed line represents the experiment in the absence of lasalocid-A. Alterations in $\Delta\psi$ (A) and medium pH (B) were followed with specific electrodes.

Na^+-dependent process were similar in the presence and absence of lasalocid-A.

The preceding experiments and others showing that nigericin, at concentrations near 20 nM, produced similar results (not shown) might indicate that lasalocid-A inhibits the putative $Ca^{2+}/2H^+$ exchange by collapsing ΔpH across the inner mitochondrial membrane. In order to test this possibility, we studied the effect of lasalocid-A on the rates of Ca^{2+} efflux under experimental conditions in which a large variation of ΔpH was caused by the antibiotic (Fig. 6) and under conditions in which ΔpH was negligible due to the presence of a high concentration of potassium acetate (Fig. 7). Fig. 6A shows that the membrane potential of liver mitochondria was not restored after Ca^{2+} addition in the

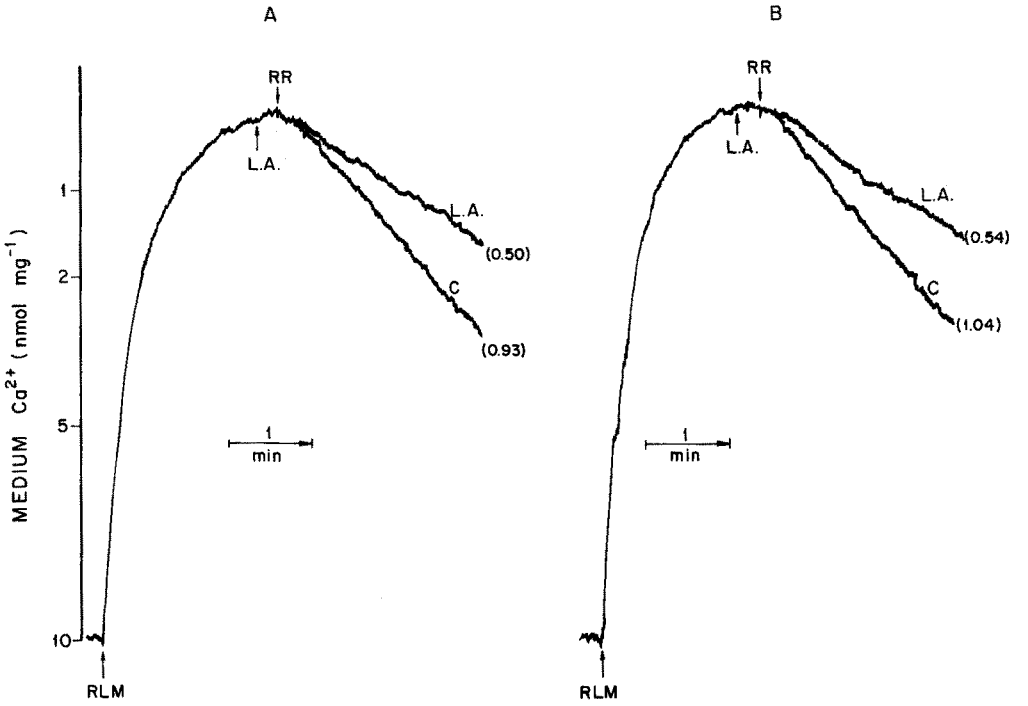

Fig. 8. Effect of lasalocid-A on Ruthenium-red-induced Ca^{2+} efflux from liver mitochondria incubated in the absence (A) or presence (B) of acetate. The experimental conditions were exactly as in Fig. 1, except that 30 mM KAc was present in (B) and the medium Ca^{2+} concentration was 10 μM. The numbers in brachets represent the rates of Ca^{2+} efflux in nmol Ca^{2+}/mg protein.

presence of NEM [31,32]. Under these conditions the addition of lasalocid-A caused regeneration of $\Delta\psi$ due to decrease in ΔpH induced by the exchange of internal K^+ for external H^+. This interpretation is supported by the experiment of Fig. 6B showing acidification of the reaction medium during Ca^{2+} uptake and alkalinization

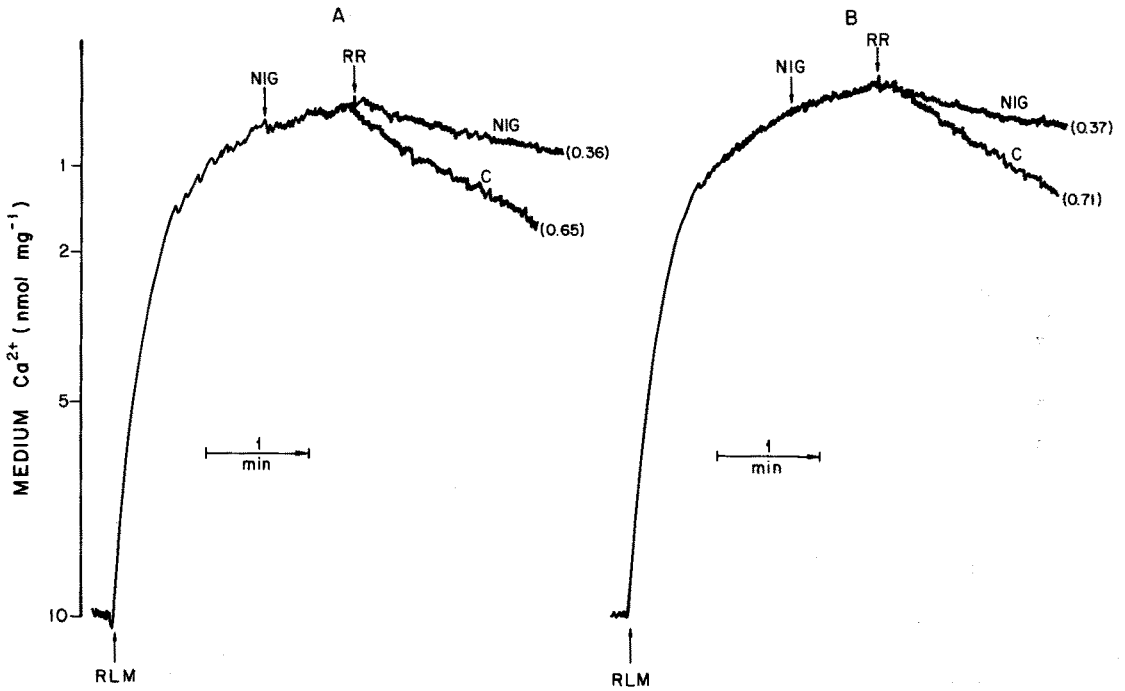

Fig. 9. Inhibition by nigericin of Ruthenium-red-induced Ca^{2+} efflux from liver mitochondria incubated in the absence (A) or presence (B) of acetate. The experimental conditions were exactly as in Fig. 8. Nigericin (20 nM), when present, was added where indicated by the arrows.

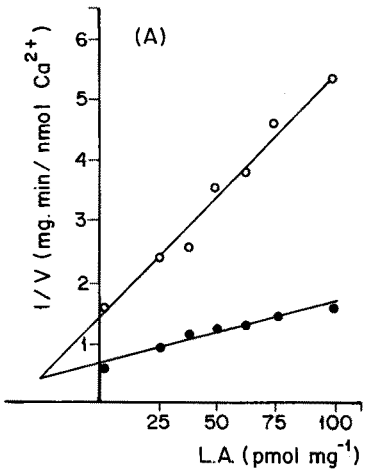

Fig. 10. Dixon plot of the rates of Ca^{2+} efflux as a function of lasalocid-A concentration. Lasalocid-A (25 to 100 pmol/mg), at two mitochondrial Ca^{2+} loads: 10 nmol Ca^{2+}/mg (○———○) and 20 nmol Ca^{2+}/mg (●———●), were used under the conditions of Fig. 8B.

after lasalocid-A addition. The value of ΔpH before lasalocid-A addition was estimated to be about 0.55 units. This was obtained using the value of 203 mV for $\Delta\tilde{\mu}_{H^+}$ when ΔpH was completely collapsed by lasalocid-A or nigericin. In the presence of acetate the changes in $\Delta\psi$ and medium pH caused by lasalocid-A after Ca^{2+} uptake were very small. This indicates that ΔpH across the inner membrane was negligible (Fig. 7A and B).

The experiments in Fig. 8A and B show that lasalocid-A causes about the same degree of inhibition of Ruthenium-red-induced Ca^{2+} efflux, regardless of the presence or not of acetate, indicating that the effect of the antibiotic is independent of changes in ΔpH. The inhibition of Ca^{2+} efflux by lasalocid-A was respectively 46 and 48% in the absence (A) and presence (B) of acetate. The experiments in Fig. 9A and B show that nigericin caused similar results.

These findings rule out the possibility that the antibiotic decreases the activity of the putative $Ca^{2+}/2H^+$ antiporter by decreasing ΔpH across the membrane and may indicate that lasalocid-A interacts directly with the Ca^{2+} efflux carrier. In fact, a Dixon plot (Fig. 10) of the reciprocal efflux velocities against lasalocid-A concentrations at constant values of $\Delta\psi$ and ΔpH and at two different Ca^{2+} loads (10 and 20 nmol Ca^{2+}/mg protein) indicates that lasalocid-A competitively inhibits Ca^{2+} efflux with a K_i of 30 pmol/mg protein.

At higher Ca^{2+} loads, lasalocid-A promotes the retention of Ca^{2+} even when the mitochondria are incubated in the presence of Ca^{2+} releasing agents such as *t*-butylhydroperoxide (Fig. 11A) and phosphate (Fig. 11B). Fig. 12 shows that when lasalocid-A is present under these conditions mitochondria can retain high membrane potentials, whereas without it a rapid decrease in $\Delta\psi$ occurs after a short period of incubation. Very similar results were obtained with both lasalocid-A or nigericin in the presence of other Ca^{2+}-releasing agents such as oxaloacetate, acetoacetate and diamide (not shown).

Discussion

The data of Fig. 1 suggest that the effects of lasalocid-A on Ca^{2+} release from mitochondria can be grouped into three categories depending on the range of

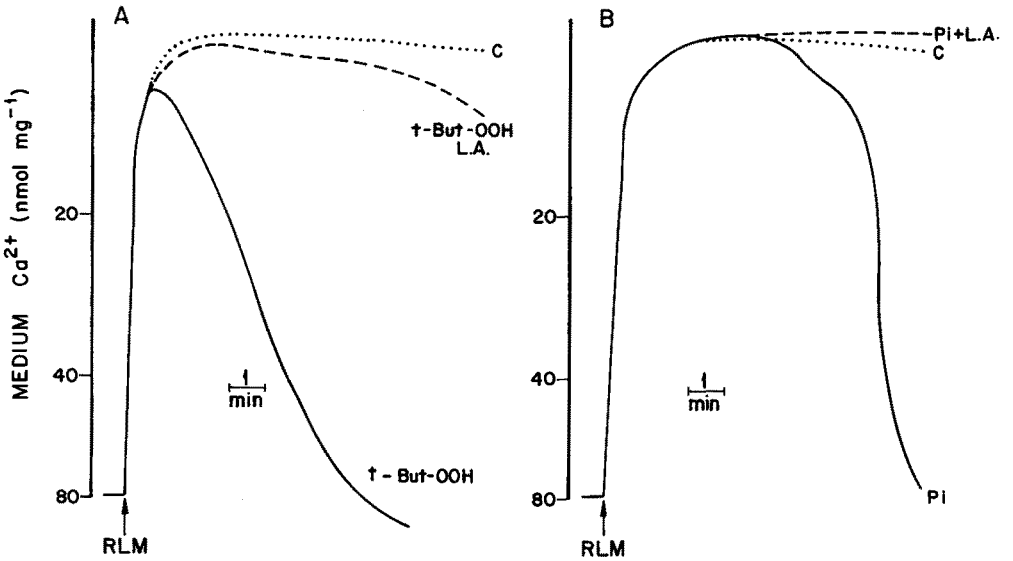

Fig. 11. Inhibition by lasalocid-A of Ca^{2+} release from liver mitochondria incubated in the presence of Ca^{2+}-releasing agents. Mitochondria (1 mg) were incubated in 1 ml of the basic medium in the absence of BSA and Mg^{2+} but containing 80 nmol Ca^{2+} and 100 μM *t*-butylhydroperoxide (A) or 3 mM Pi (B). Mitochondria were added to the medium in the absence of lasalocid-A and Ca^{2+} releasing agents (·····), in the presence of Ca^{2+}-releasing agents only (———) or in the presence of Ca^{2+}-releasing agents plus 250 pmol lasalocid-A (— — —).

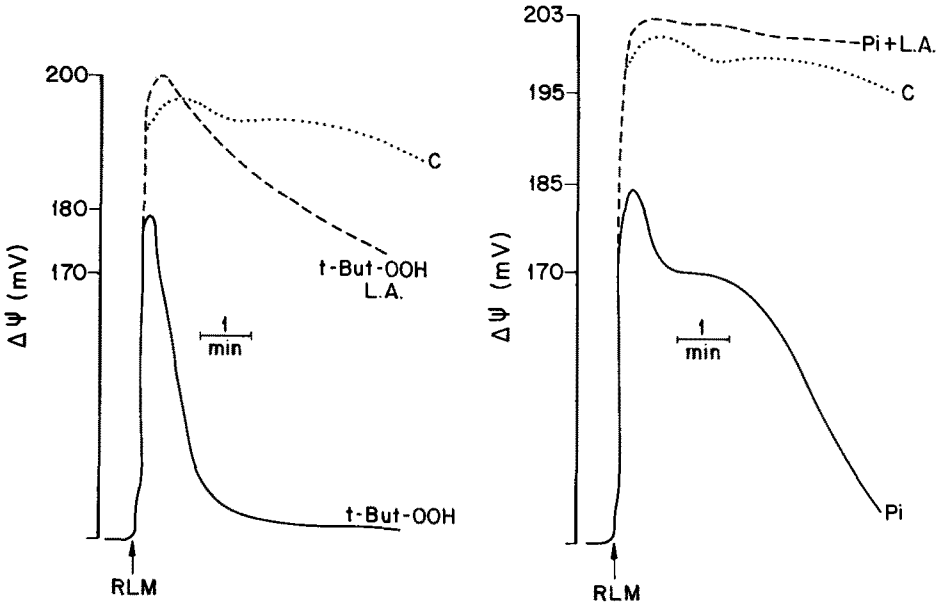

Fig. 12. Effect of lasalocid-A on $\Delta\psi$ of liver mitochondria incubated in the presence of Ca^{2+} and Ca^{2+}-releasing agents. The experimental conditions were exactly as in Fig. 11, plus 3 μM TPP^+.

the Ca^{2+} load. In an intermediate range (70–110 nmol Ca^{2+}/mg mitochondrial protein) lasalocid-A significantly stimulated Ca^{2+} release from mitochondria. At lower and higher loads lasalocid-A inhibited Ca^{2+} release, although with clearly different characteristics for this two extremes.

Inhibition of Ca^{2+} efflux at low Ca^{2+} loads

The kinetic data indicate that lasalocid-A competitively inhibits the Na^+-independent Ca^{2+} release at low Ca^{2+} loads. This and the results showing that lasalocid-A also inhibits Ca^{2+} efflux at very low ΔpH favour the interpretation of a direct interaction between the antibiotic and the efflux Ca^{2+} carrier, supporting the results ruling out an indirect mechanism dependent on alterations in ΔpH and or $\Delta\psi$. In addition, the experiments of Fig. 8 and 9, indicating that acetate, which collapses ΔpH, does not affect the rate of Ruthenium-red-induced Ca^{2+} efflux supports the idea that the mechanism of mediated Ca^{2+} efflux is not a Ca^{2+}/nH^+ exchange. The similarity of the effects of lasalocid-A on the rates of Na^+-independent Ca^{2+} release in liver and heart mitochondria and the lack of effect on the Na^+-dependent release in heart mitochondria support the conclusions [33,34] that the Na^+-independent and -dependent pathways are different entities and that in heart mitochondria the Na^+-independent mechanism does not represent a residual component of the Na^+-dependent pathway.

Effect of lasalocid-A at intermediate Ca^{2+} loads

Lin and Kun [28] showed that lasalocid-A in the range of concentrations used in this study causes dissociation of membrane-bound Mg^{2+} if ATP and Mg^{2+} are not present in the incubation medium. It is also known that the loss of membrane-bound Mg^{2+} is associated with an increase in membrane permeability to Ca^{2+} and that ATP plus Mg^{2+} protect against it [14,35–38]. The increase in the rates of Ca^{2+} release that occurs at intermediate Ca^{2+} loads may therefore be related to an increase in membrane permeability caused by the loss of membrane-bound Mg^{2+}, a process that is potentiated by lasalocid-A [28]. The presence of ATP and Mg^{2+} inhibits this effect of lasalocid-A, in agreement with the results of Lin an Kun [28]. If these interpretations are correct it can be concluded that the threshold Ca^{2+} load at which Ca^{2+} release shifts from a mediated to a mixed mediated versus nonspecific process depends on the maintenance or not of a pool of membrane-bound Mg^{2+}.

Inhibition of Ca^{2+} release at high loads

The experiments performed with mitochondria loaded with higher Ca^{2+} levels indicate that in addition to the release of membrane-bound Mg^{2+} other alterations in the membrane structure, which can be prevented by lasalocid-A, would be responsible for the higher rates of Ca^{2+} release as can be noted in the experiments of Figs. 1 and 2. This property of lasalocid-A favours the retention of Ca^{2+} by mitochondria even in the presence of Ca^{2+}-releasing agents such as phosphate, oxidants of matrix pyridine nucleotides or membrane thiols as shown in Fig. 11.

In a preceding communication [15] we presented evidence that the process of membrane permeabilization by Ca^{2+} plus a Ca^{2+}-releasing agent is dependent on the binding of the cation to sites located on the matrix face of the membrane. Since lasalocid-A is known to

dissociate Mg^{2+} [28] and possibly Ca^{2+} from the mitochondrial membrane, it might be possible that lasalocid-A favours Ca^{2+} retention into liver mitochondria by displacing the cation from the binding sites involved in the mechanism of membrane permeabilization.

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