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Salinomycin Effects on Mitochondrial Ion Translocation and Respiration

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The effects of salinomycin on alkali cation transport and membrane functions in rat liver mitochondria have been investigated. After potassium uptake, stimulated by valinomycin or monazomycin in the presence of adenosine 5'-triphosphate, salinomycin caused rapid release of K^+ from mitochondria. Salinomycin reversed valinomycin- or monazomycin-induced oscillatory swelling of mitochondria preloaded with K^+, Rb^+, and Na^+ but was without effect on Li^+ or Cs^+ preloaded mitochondria. Salinomycin blocked the retention of K^+ more effectively than the retention of Rb^+ or Na^+. Salinomycin inhibited both coupled and uncoupled respiration with strict substrate specificity in medium of low but not in high K^+ concentration. The oxidation of glutamate, α-ketoglutarate, and malate plus pyruvate was inhibited by salinomycin, but that of β-hydroxybutyrate or succinate was not significantly affected. Salinomycin inhibited adenosine triphosphatase activity of mitochondria induced by valinomycin or monazomycin in K^+ and Rb^+ medium without significantly affecting adenosine triphosphatase activity in Li^+, Na^+, or Cs^+ medium. Oxidative phosphorylation in mitochondria was inhibited by salinomycin but the inhibitory effect of salinomycin lacked the substrate specificity observed for respiration. It is proposed that salinomycin perturbs mitochondrial functions by acting as a mobile carrier for alkali cations through membranes.

Salinomycin is a monocarboxylic polyether antibiotic with antimicrobial and anticoccidial properties (14, 22). Salinomycin shows great preferential complexation with monovalent over divalent cations in a two-phase system and mediates their transport across a CCl_4 bulk phase (23).

The ability of monocarboxylic polyether antibiotics to transport cations across biological membranes or into a bulk organic phase has been described (25, 27, 28, 30). These antibiotics carry cations through the lipid barrier of membranes as lipid-soluble cation complexes by a passive diffusion process (1, 32). Pressman demonstrated the cation discrimination patterns of certain ionophorous antibiotics by measuring complexability with cations in a two-phase distribution system (25, 27). With respect to their ability to block ion translocation, these antibiotics exhibit various effects on membrane systems of mitochondria (6, 8, 11), sarcoplasmic reticulum (2, 17), lymphocytes (19, 20), platelets (7, 21), chloroplasts (33), and microbes (4, 11). Thus these ionophorous antibiotics have served as valuable tools for studying dynamic properties of ion-carrier mechanisms in biological membranes.

In the present study, we describe the effect of salinomycin on alkali cation transport and membrane functions in rat liver mitochondria.

MATERIALS AND METHODS

Salinomycin and monazomycin were prepared from the culture broths of *Streptomyces* strain no. 80614 and no. 3682-JTtl, respectively. Valinomycin was purchased from P-L Biochemical Inc., Milwaukee, Wis., tris(hydroxymethyl)aminomethane (Tris)-adenosine 5'-triphosphate (ATP), and adenosine 5'-diphosphate (ADP) (free from salt) from Sigma Chemical Co., St. Louis, Mo., and [^{32}P]phosphoric acid from Japan Atomic Energy Research Institute, Tokyo, Japan. Substrates and nucleotides were adjusted to pH 7.4 with triethanolamine and the antibiotics were dissolved in ethanol.

Mitochondria were prepared from livers of male rats, weighing 200 to 250 g, according to Johnson and Lardy (13) and then suspended in 250 mM sucrose solution.

The concentration of K^+ was monitored with an Orion K^+-specific electrode (92-19).

Respiration was monitored polarographically with a Clark-type oxygen electrode.

Mitochondrial swelling was monitored at 515 nm by light-scattering changes by means of a Hitachi 333 recording spectrophotometer.

Adenosine triphosphatase (ATPase) activity was

determined by measuring inorganic phosphate release during 5-min incubations at 30 C (34).

Oxidative phosphorylation was measured by determining the amount of ^{32}P incorporated into ATP according to Nielsen and Lehninger (24).

Mitochondrial protein concentrations were measured by the method of Lowry et al. (18).

RESULTS

Effect of salinomycin on alkali metal cation transport and light-scattering changes induced by valinomycin or monazomycin in rat liver mitochondria. Valinomycin and monazomycin are known to stimulate mitochondrial uptake of cations, a process which is accompanied by oscillatory mitochondrial swelling (3, 6, 15, 26).

When potassium uptake was stimulated by valinomycin (10^{-7} M) or monazomycin (10^{-7} M) in the presence of ATP, the addition of salinomycin (4×10^{-7} M) caused rapid release of K^+ and reversed oscillatory mitochondrial swelling (Fig. 1A).

Pretreatment with salinomycin (4×10^{-7} M) prevented valinomycin (10^{-7} M) and monazomycin (8×10^{-8} M) from inducing K^+ transport and oscillatory swelling.

When uptake of alkali cations was stimulated by valinomycin in the presence of ATP, glutamate, or succinate, salinomycin reversed mitochondrial oscillatory swelling linked to K^+ or Rb^+ transport but was without effect on the swelling coupled to Cs^+ transport (Fig. 1B). A similar reversal of mitochondrial swelling by salinomycin was observed when monazomycin induced mitochondrial swelling resulting from cation uptake in the presence of ATP, glutamate, or succinate (Fig. 1B). Salinomycin produced contraction of mitochondria preloaded with K^+, Rb^+, or Na^+, but not with Li^+ or Cs^+. Salinomycin was more effective in blocking the retention of K^+ than Rb^+ or Na^+ (Fig. 1B).

Effect of salinomycin on substrate oxidation in mitochondria. Salinomycin caused inhibition of respiration proportional to the increase in redox potential induced by the substrate. In low K^+ medium, salinomycin partially inhibited state 4 respiration (in the presence of substrate but absence of ADP) with glutamate, α-ketoglutarate, or malate plus pyruvate but did not affect that with β-hydroxybutyrate or succinate (Table 1). The inhibition of state 4 respiration induced by salinomycin was restored in the presence of a high potassium concentration in the medium (Table 1). ADP-activated respiration (state 3) with glutamate, α-ketoglutarate, or malate plus pyruvate as substrates was significantly inhibited by salinomycin but that with β-hydroxybutyrate or succinate was only slightly inhibited.

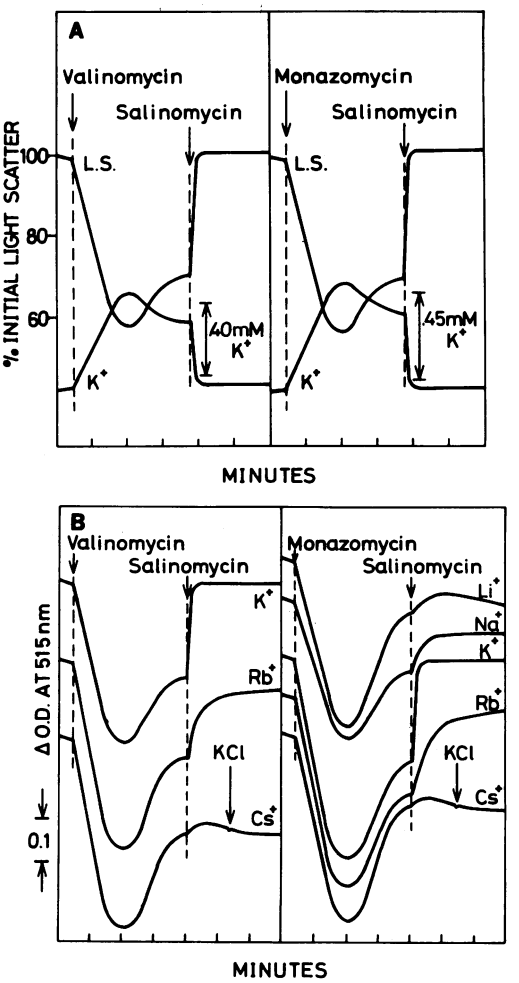

FIG. 1. *(A) Effect of salinomycin on potassium transport and light scattering changes induced by valinomycin or monazomycin in liver mitochondria. A downward deflection of the light scattering trace (L.S.) is associated with swelling of mitochondria. An upward deflection in the K^+ trace represents a decrease of its concentration in the medium or uptake of K^+ by mitochondria. The reaction system contained 20 mM triethanolamine-Cl, 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 3.8 to 4.8 mg of mitochondrial protein. Final volume, 5 ml (pH 7.3); temperature, 27 C. Antibiotic additions at indicated points: 10^{-7} M valinomycin; 10^{-7} M monazomycin; 4×10^{-7} M salinomycin. (B) Effect of salinomycin on ATP-dependent swelling of mitochondria resulting from uptake of alkali metal cations induced by valinomycin or monazomycin. The reaction system contained 20 mM Tris-acetate, pH 7.4; 5 mM $MgCl_2$; 30 mM alkali metal cation (Cl salt); 3 mM Tris-ATP; 150 mM sucrose; and 0.1 to 0.2 mg of mitochondrial protein. Final volume, 3 ml (pH 7.4); temperature, 27 C. Valinomycin was added at a concentration of 10^{-7} M, monazomycin at 8×10^{-8} M, and salinomycin at 4×10^{-7} M.*

TABLE 1. *Inhibition of substrate-dependent respiration by salinomycin*[a]

Substrate	Inhibition (%) of respiration		
	State 4		State 3
	20 mM K^+	75 mM K^+	20 mM K^+
Glutamate	25	3	100
α-Ketoglutarate	24	4	100
Malate + pyruvate	19	4	54
β-Hydroxybutyrate	0	−1	17
Succinate	−15	−18	8

[a] The reaction mixture contained 10 mM Tris-chloride, pH 7.4; 10 mM Tris-PO_4, pH 7.4, 5 mM $MgCl_2$; 160 mM sucrose with 20 mM KCl, or 100 mM sucrose with 75 mM KCl; 5 mM substrate; and 2 to 3 mg of mitochondrial protein. Final volume, 3 ml (pH 7.4); temperature, 25 C. Salinomycin was added at a concentration of 4×10^{-6} M for state 4 respiration and 1.3×10^{-6} M for ADP-activated respiration.

Salinomycin also inhibited uncoupled respiration, stimulated by valinomycin, monazomycin, or 2,4-dinitrophenol (DNP) with the same substrate specificity seen with coupled respiration (Table 2). Oxidation of glutamate or α-ketoglutarate in the presence of uncoupling agents was markedly inhibited, whereas that of malate plus pyruvate was only slightly inhibited. Salinomycin slightly stimulated the oxidation of β-hydroxybutyrate or succinate in uncoupled mitochondria (Table 2).

Figure 2a shows the effect of various cations accumulated by valinomycin or monazomycin on the inhibition of glutamate oxidation by salinomycin. The addition of salinomycin inhibited glutamate oxidation in mitochondria previously loaded with K^+, Rb^+ or Na^+, but not with Li^+ or Cs^+. The inhibition induced by salinomycin at low and moderate K^+ concentrations was overcome by a high K^+ concentration (81% inhibition at 1 to 20 mM K^+ concentration was reduced to 73 and 49% in the presence of 50 and 75 mM K^+, respectively).

Effect of various cations on the inhibition by salinomycin of mitochondrial ATPase induced by valinomycin or monazomycin. Salinomycin inhibited ATPase activity induced by valinomycin or monazomycin in K^+ and Rb^+ medium without significantly affecting ATPase activity in Li^+, Na^+, or Cs^+ medium (Fig. 2b).

Effect of salinomycin on oxidative phosphorylation in mitochondria. Salinomycin significantly inhibited oxidative phosphorylation and the inhibition seemed to lack the substrate specificity that was observed for respiratory inhibition by salinomycin (Table 3). Oxidative phosphorylation was more susceptible to the antibiotic than was ADP-activated respiration.

DISCUSSION

Mode of action studies with monocarboxylic polyether antibiotics have revealed that these antibiotics form hydrophobic complexes with cations and catalyze electroneutral exchanges of intracellular cations for extracellular protons across various membranes (25, 27-29, 31, 32).

After potassium uptake had been stimulated by valinomycin or monazomycin, salinomycin addition caused rapid release of K^+ from mitochondria and reversed oscillatory swelling (Fig. 1). Salinomycin reversed the swelling of mitochondria preloaded with K^+, Rb^+, and Na^+ by valinomycin or monazomycin, but was without effect on the swelling induced by Li^+ or Cs^+ (Fig. 1). Salinomycin was more effective in blocking the retention of K^+ than the retention of Rb^+ or Na^+. These results suggest that salinomycin produces contraction of mitochondria through loss of accumulated alkali cations. Salinomycin inhibited the oxidation of glutamate, α-ketoglutarate, or malate plus pyruvate, but not of β-hydroxybutyrate or succinate (Tables 1 and 2). The respiratory inhibition induced by salinomycin was overcome by high K^+ concentration in the medium. Salinomycin inhibited glutamate oxidation in mitochondria preloaded with K^+, Rb^+, or Na^+, but not with Li^+ or Cs^+ (Fig. 2a).

These results are consistent with the possibility that the respiratory inhibition by salinomycin may be the result of loss of cations in mitochondria. It is known that under certain conditions, internal acidification, and K^+ loss in mitochondria interfere with anionic substrate permeability (8-10). This interference is more significantly demonstrated using DPN-linked substrates than it is using β-hydroxybutyrate or succinate (5, 6, 11, 31).

Salinomycin inhibited ATPase activity in the presence of valinomycin or monazomycin in K^+ or Rb^+ medium without significantly affecting

TABLE 2. *Effect of salinomycin on substrate oxidation stimulated by various uncoupling agents*

Substrate	Inhibition (%)		
	Valinomycin	Monazomycin	DNP
Glutamate	79	81	94
α-Ketoglutarate	74	75	90
Malate + pyruvate	5	4	8
β-Hydroxybutyrate	−35	−15	−20
Succinate	−5	−8	−15

[a] The reaction mixture was the same as described in Table 1. Respiration was previously stimulated by the addition of valinomycin (10^{-7} M), monazomycin (10^{-7} M), or DNP (2×10^{-5} M), then salinomycin was added at a concentration of 1.3×10^{-6} M.

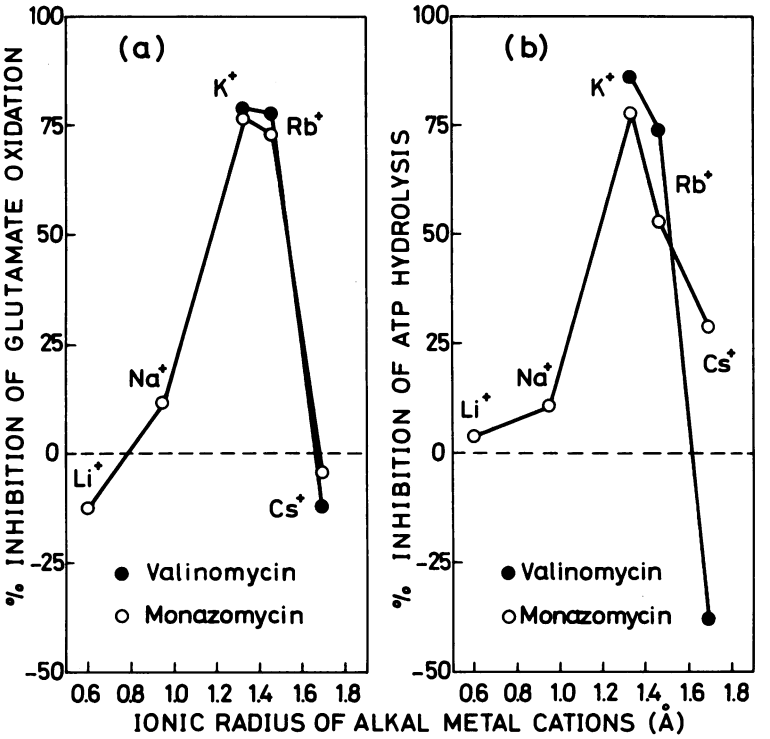

FIG. 2. *(a) Effect of different alkali metal cations on the inhibition by salinomycin of glutamate oxidation stimulated by valinomycin or monazomycin. The reaction system contained 10 mM Tris-chloride, pH 7.4; 10 mM Tris-PO_4, pH 7.4; 5 mM $MgCl_2$; 20 mM alkali metal cation (Cl salt); 160 mM sucrose; and 2 to 3 mg of mitochondrial protein. Final volume, 3 ml (pH 7.4); temperature, 25 C. Glutamate oxidation was previously stimulated by the addition of valinomycin (10^{-7}M) or monazamycin (10^{-7}M), then salinomycin was added at a concentration of 1.3 × 10^{-6}M. (b) Effect of various alkali metal cations on the inhibition by salinomycin of mitochondrial ATPase stimulated by valinomycin or monazomycin. The reaction system contained 10 mM triethanolamine-chloride, pH 7.4; 10 mM acetate-triethanolamine, pH 7.4; alkali metal cations (Cl salt); 3 mM Tris-ATP; 100 mM sucrose; and 0.3 mg of mitochondrial protein in a final volume of 1 ml (pH 7.4). Incubation was conducted for 5 min at 30 C. Valinomycin was added at a concentration of 10^{-7}M, monazomycin at 8 × 10^{-8}M, and salinomycin at 1.3 × 10^{-6}M.*

ATPase activity in Li^+, Na^+, or Cs^+ medium (Fig. 2b).

The data suggest that salinomycin like the antibiotics nigericin and the monensins has the ability to carry cations across mitochondrial membranes (6, 8, 11). These antibiotics form lipid-soluble complexes with alkali cations and act as mobile carriers for cations through lipid barriers of membranes (8, 25, 27). In our previous study, we showed that salinomycin transports monovalent cations more effectively than divalent cations from aqueous buffer into an organic solvent in an organic solvent-water, two-phase distribution system (23). The sequence of ion selectivity of salinomycin is as follows: $K^+ > Na^+ > Cs^+ > Sr^{2+} > Ca^{2+}, Mg^{2+}$. Salinomycin therefore resembles nigericin with respect to its cation specificity (23, 27). Nigericin, however, has been found to uncouple oxidative phosphorylation in mitochondria at high concentrations (8), whereas salinomycin does not stimulate respiration or ATPase activity in miochondria at the concentrations tested (Table 1 and Fig. 2b). On the other hand, when ATP-dependent swelling of mitochondria resulting from Rb^+ uptake was induced by valinomycin, salinomycin produced contraction of mitochondria (Fig. 1), whereas the monensins do not (6). The reasons for these differences in activity between salinomycin and other antibiotics are not clear but may be due to differences in their cation discrimination patterns.

Salinomycin markedly inhibited oxidative phosphorylation in mitochondria but the inhibitory effect lacked substrate specificity (Table 3). The stimulatory effect of monovalent cations, especially of K^+, on oxidative phosphorylation has been well described (9, 10). It is sug-

TABLE 3. *Effect of salinomycin on oxidative phosphorylation in mitochondria*[a]

Substrate	ATP synthesis (nmol/mg of protein per min)		Inhibition %
	Control	Salinomycin	
Glutamate	115	0	100
α-Ketoglutarate	93	0	100
Malate + pyruvate	96	5	94
β-Hydroxybutyrate	134	36	73
Succinate	161	44	73

[a] The reaction system contained 10 mM Tris-chloride, pH 7.4; 5 mM $MgCl_2$; 25 mM KCl; 150 mM sucrose; 10 mM substrate; 1.3 nmol of salinomycin; and 1.3 mg of mitochondrial protein. Final volume, 1 ml (pH 7.4); temperature, 30 C. Mitochondria were preincubated for 1 min with or without 1.3 nmol of salinomycin before 2.5 mM ADP and 2.5 mM potassium phosphate (pH 7.4) labeled with ^{32}P were added. After a 1-min reaction, the initial rate of ATP synthesis was determined by the amount of ^{32}P incorporated into ATP.

gested that the inhibitory effect of salinomycin on oxidative phosphorylation may be the result of loss of cations, particularly K^+, in mitochondria.

Recently, we found that an inhibitory effect of salinomycin on bacterial growth of *Bacillus subtilis* and *Staphylococcus aureus* was reversed by high concentrations of alkali metal cations in the growth media (manuscript in preparation). These results are consistent with present data showing that the inhibitory effects of salinomycin on mitochondria were reversed by the addition of high concentrations of alkali metal cations.

In conclusion, the effect of salinomycin on mitochondria may be attributable to its monovalent cation carrying activity like other monovalent cation ionophores.

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