EFFECT OF PRIMYCIN ON THE INNER MEMBRANE PERMEABILITY OF RAT LIVER MITOCHONDRIA

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The effects of primycin on mitochondrial respiration, volume changes, ATPase activity and the acidification following ATP hydrolysis were studied. Primycin in concentrations below 2~3 nmoles/mg mitochondrial protein reacts only with energized mitochondria rendering their inner membrane permeable to K^+, Na^+, $Tris^+$ but not to TEA^+. Above this concentration primycin interacts both with energized and deenergized mitochondria and the inner membrane also becomes permeable for H^+, Cl^- but not for ATP. In this case mitochondria very probably lose Mg^{2+}.

It is concluded that primycin up to concentrations of 2~3 nmoles/mg mitochondrial protein acts like an ionophore, while at higher concentrations it changes the permeability properties of the mitochondrial inner membrane without a drastic alteration of the membrane itself.

Primycin was isolated and found to inhibit the growth of Gram-positive bacteria by VÁLYI-NAGY *et al.*[1]. Its structure (Fig. 1) was established by ABERHARDT *et al.*[2] and FEHR *et al.*[3]. The mechanism of primycin action was ascribed to an inhibition of the DNA-dependent RNA synthesis[4,5]. However, HORVÁTH *et al.*[6] could not find a selective inhibition of RNA synthesis in Gram-positive bacteria by primycin. On the other hand, they have shown that an effect on the bacterial cell membrane is responsible for the bacteriostatic action of primycin. This membrane effect prompted us to see whether primycin interacts also with mitochondria. This paper deals with the effect of primycin on the inner membrane of rat liver mitochondria. It will be shown that primycin characteristically alters the permeability properties of this membrane.

Materials and Methods

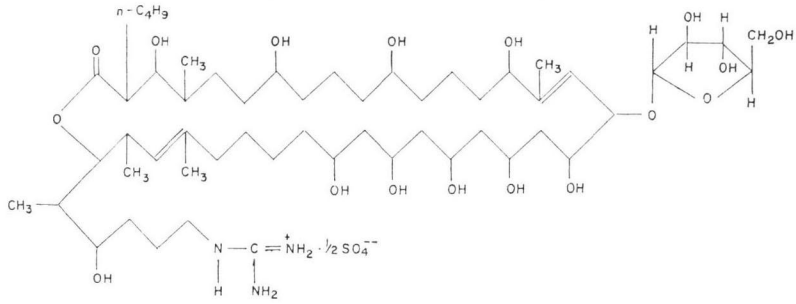
Rat liver mitochondria were prepared according to SCHNEIDER[7] in a medium containing 250 mM sucrose, 1 mM EGTA and 4 mM Tris-HCl buffer pH 7.2 as described previously.[8]

Oxygen uptake was measured with a Clark-type oxygen electrode. Acidification of the media following ATP hydrolysis was monitored with a Radiometer (Copenhagen) pH-meter using a G202C type glass electrode. ATPase activity of mitochondria was measured by the method of WEINER and LARDY[9] as described by KÖNIG *et al.*[8]. Swelling of mitochondria was followed in a Specord UV Vis spectrophotometer (Carl-Zeiss, Jena) monitoring the changes of optical density at 610 nm. All measurements were carried out at 25°C and pH 7.2 in 3 ml volume. The composition of media used and further experimental details are given in the legends to figures and tables.

The protein content of mitochondrial preparations was determined according to SCHACTERLE and

Abbreviations: EGTA, ethyleneglycol bis(-aminoethylether)-N,N′-tetraacetic acid; TMPD, N,N,N′,N′-tetramethyl-*p*-phenylenediamine; TEA, triethanolamine; DNP, 2,4-dinitrophenol; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethane sulphonic acid; cAtr, carboxyatractyloside; TRIS, Tris-(hydroxymethyl)aminomethane.

Fig. 1. The structure of primycin.



POLLACK[10] using bovine serum albumin (Sigma) as standard.

The chemicals used were the purest commercially available. ATP (disodium salt) was converted to triethanolammonium salt by using Dowex 50×1 resin at 0°C. cAtr was purchased from Boehringer (Mannheim, Germany), rotenone from Serva (Heidelberg, Germany). Valinomycin was kindly supplied by A. S. KHOKHLOV, Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences. Primycin was obtained from Chinoin Pharmaceutical Works (Budapest) and dissolved in methanol or propylene glycol. Control experiments showed that even the greatest amount of solvents used were without any effect on mitochondria.

Results

Primycin was found to increase the state 4 respiration of mitochondria. Dose curve of the initial rate values of primycin stimulated respiration showed a maximum at 2~3 nmoles/mg mitochondrial protein (Fig. 2). However, above that concentration, stimulation gradually decreased in time. The effect turned to an inhibition in the presence of glutamate+malate or succinate as substrates, but not if the substrates were TMPD+ascorbate (Fig. 3). Both the period to develop the inhibition of respiration and the dose of primycin required for maximal stimulation of respiration were increased by decreasing the concentration of KCl in the incubation medium. Added Mg^{2+} did not influence at all the above mentioned effects of primycin on respiration (not shown).

In accordance with its stimulatory effect on state 4 respiration primycin also activated the "basal ATPase activity" (*i.e.* measured in the absence of uncoupler) of mitochondria. In the absence of Mg^{2+} the primycin dose dependence curve of the stimulation of basal ATPase activity showed a maximum, whereas in the presence of Mg^{2+} the curve was found to be of the saturation type (Fig. 4). The amount of primycin to elicit maximal stimulation of the basal ATPase activity—similarly to that of stimulating state 4 respiration—could be increased by decreasing the concentration of KCl in the medium (see also Fig. 7). The stimulation by primycin of both state 4 respiration and basal ATPase activity indicates that this antibiotic increases

Fig. 2. Effect of primycin on the state 4 respiration in different concentrations.

The reaction mixture contained 90 mM sucrose, 80 mM KCl, 0.1 mM EGTA, 5 mM TEA-phosphate, 5 mM TEA-glutamate, 1.7 mM TEA-malate and mitochondria (3.3 mg protein). The experimental points were calculated from the initial rate values of oxygen uptake.

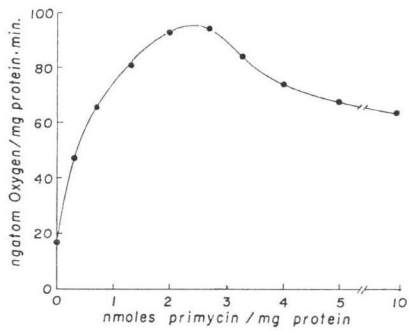


Fig. 3. Effect of primycin on the state 4 respiration in the presence of different substrates.

The medium was identical with that of Fig. 2 except that TEA-glutamate (5 mM) and TEA-malate (1.7 mM) were replaced by TEA-succinate (4 mM) in exp. b and by TMPD (0.6 mM) and TEA-ascorbate (6 mM) in exp. c. 2.5 mg of mitochondrial protein (Mt.) and 4 nmoles/mg protein of primycin (Pr.) were added where indicated. The numbers represent oxygen uptake expressed in n atoms/mg mitochondrial protein × min.

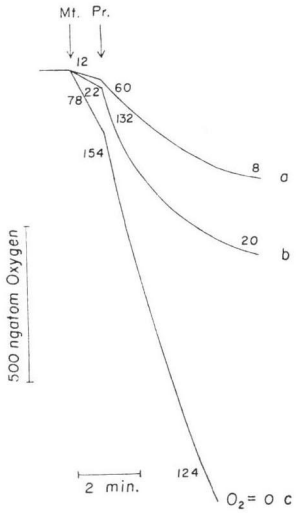


Fig. 4. Effect of primycin on the basal ATPase activity of mitochondria.

Experimental conditions were the same as those of Fig. 2 except that respiratory substrates and phosphate were not present and the medium contained also TEA-ATP (6 mM) in the presence of Mg^{2+} (3.3 mM), and in the absence of Mg^{2+}. The reaction was started with mitochondria (3.1 mg protein) and was stopped by addition of ice cold trichloroacetic acid. The inorganic phosphate liberated was determined.

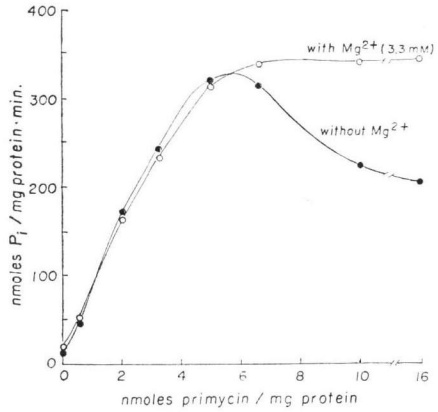
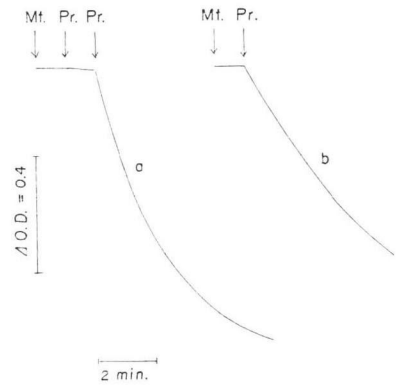


Fig. 5. Passive swelling of mitochondria induced by primycin.

The medium contained 140 mM KNO_3, 0.1 mM EGTA, 5 mM TEA-NO_3, 5 mM TEA-glutamate and 1.7 mM TEA-malate as substrates.

In exp. a, rotenone (6 μM) was also present. The amount of mitochondrial protein used was 3.1 mg in each experiment. Each addition of primycin was 1.5 nmoles/mg protein.



the permeability of the mitochondrial inner membrane for cations or protons or for both.

As can be seen in Fig. 5a, 3 nmoles primycin/mg mitochondrial protein induced a high amplitude (passive) swelling of non-respiring mitochondria suspended in the isoosmotic solution of the penetrant NO_3^- anion[11] and otherwise non-penetrant K^+ cation. Thus, the antibiotic rendered the inner membrane permeable for K^+. On the other hand, in the presence of 1.5 nmoles primycin/mg mitochondrial protein, mitochondria did not swell under identical experimental conditions (Fig. 5a). However, if the mitochondria were energized (either by respiration or by hydrolyzing ATP) a swelling of high amplitude was induced even by 1.5 nmoles primycin/mg mitochondrial protein (Fig. 5b).

From the data of Table 1 it can be concluded that primycin rendered permeable the mitochondrial inner membrane also for Na^+ and $Tris^+$, but not for TEA^+ both under energized and deenergized conditions. It is also apparent that a Cl^- permeability was simultaneously induced by this higher

primycin dose (2~3 nmoles/mg mitochondrial protein).

In a medium of low K^+ content even 1 nmole primycin/mg mitochondrial protein induced a valinomycin-like low amplitude swelling of respiring (energized) mitochondria indicating an active K^+ uptake (Fig. 6a). The K^+ uptake induced either by primycin or valinomycin could be prevented or reversed by the addition of a higher dose of primycin, as in the case of uncouplers (protonophores) or respiratory inhibitors (Fig. 6a, b, c). Since inhibition of respiration by this dose of primycin developed after a significantly longer time than for K^+ efflux took place it seemed plausible to suppose that primycin rendered the inner membrane permeable also for protons.

Table 1. Primycin-induced passive swelling of mitochondria in isoosmotic media of different ion composition.

	Rate of swelling ($\frac{\Delta O.D. \text{ at } 610 \text{ nm}}{\text{min.}}$)	
	of respiring mitochondria	of rotenone inhibited mitochondria
140 mM KNO_3	0.21	0.45
〃 $NaNO_3$	0.21	0.40
〃 Tris-NO_3	0.24	0.26
〃 TEA-NO_3	0.00	0.02
〃 KCl	0.20	0.44
〃 NaCl	0.21	0.42
〃 Tris-Cl	0.19	0.25
〃 TEA-Cl	0.01	0.04

The media contained also 0.1 mM EGTA, 5 mM TEA-NO_3 or Cl, 5 mM TEA-glutamate and 1.7 mM TEA-malate. Where indicated rotenone (6 μM) was also present. In each experiment 2.9 mg mitochondrial protein was used. Swelling was induced by 1.5 nmoles/mg protein of primycin in the case of respiring mitochondria, or 3.3 nmoles/mg protein of primycin in the case of inhibited mitochondria, respectively.

Fig. 7 demonstrates that while the ATPase activity of mitochondria (measured by the inorganic phosphate liberated) increased monotonely with increasing the amount of primycin added to the medium, the acidification of the medium following ATP hydrolysis induced by primycin exhibited a dose dependence curve of maximum type. This finding suggests that primycin in a dose higher than 2~3 nmoles/mg mitochondrial protein in fact rendered the mitochondrial inner membrane permeable for protons.

The data of Table 2 show that mitochondrial ATPase activity remained completely carboxyactractyloside-sensitive even in the presence of a higher dose of primycin. This finding convincingly demonstrates that ATP and ADP could not cross the inner membrane *via* other route than the ATP/ADP translocase. Therefore, a profound alteration of the inner membrane does not seem to

Fig. 6. Dose dependence of primycin effect on K^+ movement.

The reaction mixture contained 245 mM sucrose, 0.2 mM EGTA, 2 mM HEPES, 0.8 mM phosphate, 2.6 mM K^+, plus 5 mM TEA-glutamate and 1.7 mM TEA-malate as substrates. The amount of mitochondrial protein used was 2.7 mg in each experiment. Further additions where indicated were DNP (200 μM), rotenone (6 μM) and valinomycin (0.2 μM).

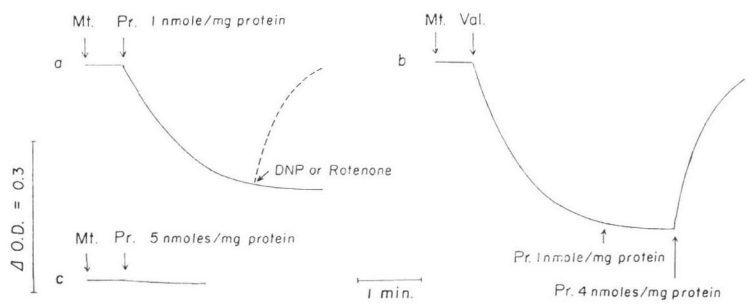


Fig. 7. Acidification of the medium following ATP hydrolysis induced by primycin.

The medium contained 230 mM sucrose, 10 mM KCl, 0.1 mM EGTA, 5 mM TEA-Cl, 6 μM rotenone, 3.3 mg mitochondrial protein and 1.2 mM TEA-ATP as substrate. The reaction was started with the substrate. The calculations were based on the pH change caused by addition of a given amount of HCl.

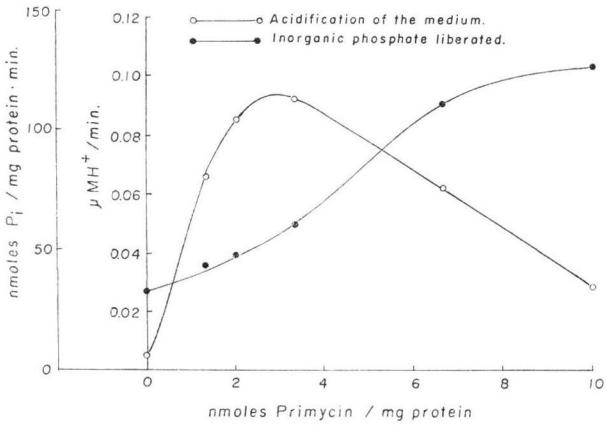


Table 2. Inhibition of primycin-stimulated ATPase activity by carboxyatractyloside

Additions	ATPase activity nmoles P_i / mg protein × min.
DNP (200 μM)	316
Primycin (4 nmoles/mg protein)	318
Primycin (10 nmoles/mg protein)	242
DNP+cAtr (15 μg/mg protein)	14
Primycin (4 nmoles/mg protein) +cAtr	17
Primycin (10 nmoles/mg protein) +cAtr	12

Experimental conditions were identical with those of Fig. 4 except that 3.2 mg mitochondrial protein was used.

occur in the presence of even a high concentration of primycin.

Discussion

The findings that primycin up to 2~3 nmoles/mg mitochondrial protein concentrations stimulates state 4 respiration, basal ATPase activity and induces swelling of energized mitochondria in the presence of otherwise impermeable cations clearly demonstrate that it acts like an ionophore. In this respect among those antibiotics which have been tested and found to act on mitochondria—according to our best knowledge—it is most similar to lienomycin[12].

Both antibiotics interact only with energized mitochondria rendering the inner membrane permeable to K^+, Na^+ and $Tris^+$, both have a basic group.

There are also some differences both in their structure and action. While lienomycin is a polyene[13], primycin has only two double bonds. However, the most striking difference between the effect of the two antibiotics is that primycin at concentrations above 2~3 nmoles/mg mitochondrial protein renders the inner membrane permeable also for protons and Cl^-. At such concentrations primycin interacts also with deenergized mitochondria. One can speculate that the strongly basic guanidino group might be responsible for these additional effects of primycin.

The inhibition following the temporary stimulation of respiration caused by higher doses of primycin is similar to that found in the simultaneous presence of both valinomycin and DNP[14,15]. Thus, the inhibition of respiration by primycin in the presence of low KCl concentration can be best explained by a substrate depletion[16] as a consequence of its combined ionophore and "protonophore" action, while at high KCl concentrations possibly Cl^- is preferentially taken up instead of the substrates. It should be emphasized that even at higher primycin doses (*i.e.* above 2~3 nmoles/mg mitochondrial protein) a profound alteration of the mitochondrial inner membrane did not occur, either electronmicroscopically or functionally, since it remained impermeable to TEA^+ and the transport of ATP through it completely sensitive to carboxyatractyloside. However, the difference in shape of the primycin dose ATPase activity curves obtained in the presence and absence of added Mg^{2+} respectively, indicates a Mg^{2+} depletion of the inner membrane. WOJTCZAK suggested the appearance of a Cl^- permeability and a K^+/H^+ exchange through the inner membrane as a consequence

of Mg^{2+} depletion[17,18]. It requires further studies to learn to what extent a primycin-induced Mg^{2+} depletion can be made responsible for the induction of proton and Cl^- permeability through the mitochondrial inner membrane.

Acknowledgements

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