MEMBRANE MEDIATED INHIBITION OF PROTEIN SYNTHESIS BY VALINOMYCIN IN RETICULOCYTES

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1. Introduction

In the past few years considerable attention has been directed to the action of Valinomycin on the K^+ ion transport mechanism [1] and less to its properties as an antibiotic [2, 3]. As the protein synthesis apparatus is known to require high K^+ concentrations, it was believed that Valinomycin acted as an antibiotic by limiting the K^+ concentration available to protein synthesis [4]. We examined this hypothesis in terms of the plasma membrane functions in order to relate their involvement in protein synthesis regulation. Evidence is provided in this work that Valinomycin acts on the elongation process in a way which does not depend primarily on the loss of K^+ ions by the cells and that its effects are membrane mediated. The reticulocyte system which has a very well defined protein synthesis machinery and whose membrane characterisitics are characterized was employed as a biological system.

2. Materials and methods

2.1. *Source of materials*

Reticulocyte enriched blood was obtained by injection of young white rabbits [5] with phenylhydrazine. The blood was collected by cannulation when levels of 60–70% reticulocytes was attained. Ribosome and S100 supernatant fractions were prepared from Valinomycin and untreated cells according to previously described procedures [6]. The KCl wash was obtained by suspending ribosome pellets in a 1 M KCl solution in 60% glycerol. This suspension was then centrifuged 4 hr at 200,000 g to eliminate any remaining ribosomes and subunits.

Valinomycin was purchased from Sigma Biochemical Corp. and dicyclohexyl-18-crown-6 (D.C.) was a gift of Dupont Co.

For incorporation experiments reticulocytes were washed three times in isotonic buffer before they were suspended in Schulmann medium [7] containing those concentrations of KCl indicated in each experiment. The [^{14}C]leucine used had a specific activity of 342 mCi/mmole. Valinomycin was dissolved in ethanol at concentrations ranging from 10^{-10} to 10^{-4} depending upon the experiment (see legends). Ethanol controls were performed in parallel experiments. Dicyclohexyl-18-crown-6 was used at 10^{-5} M to 10^{-3} M. These two products were added to the incubation mixture just prior to incubation.

The cells were agitated when incubated at 37° for time periods varying from 1 min to 2 hr.

Cell free incorporation of amino acids into polypeptides was performed according to procedures described by Allen and Schweet [8].

Polysomal fractions were analysed in a Model E Spinco analytical Ultracentrifuge and by electron microscopy [9]. The ATP content of cells was determined by the luciferin–luciferase reaction using a Tri-Carb scintillation counter.

3. Results

3.1. *Effect of valinomycin on [^{14}C]leucine incorporation by reticulocytes*

When valinomycin at concentrations of 10^{-5} M was added to reticulocytes in Schulmann medium containing 14 mM KCl, incorporation of [^{14}C]leucine into polypeptides was 90% inhibited after 2 min. This

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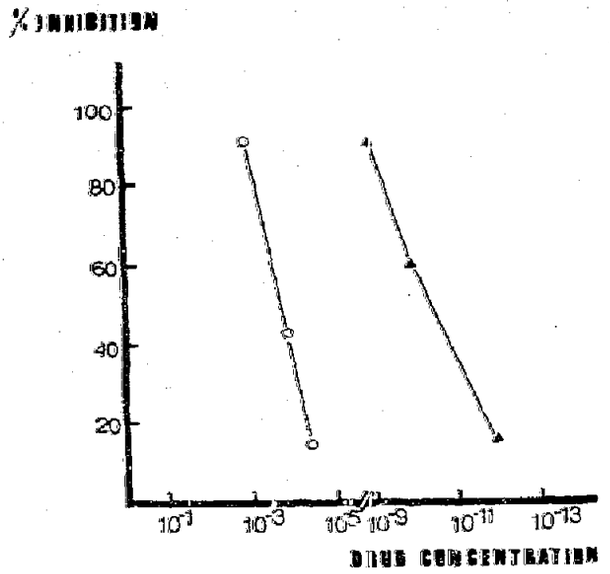

Fig. 1. 0.1 ml of reticulocyte packed cells were incubated 40 min in Schulmann medium containing [^{14}C]leucine and valinomycin (▲) or D.C. (○) at different concentrations and control. Incorporation of [^{14}C]leucine into polypeptides was measured by hot TCA precipitation and the percent of inhibition as compared to control calculated for each duplicate sample.

inhibition, which was virtually instantaneous, remained constant during longer incubation periods at nanomolar concentrations of valinomycin (see fig. 1).

Table 1
Inhibition of incorporation [^{14}C]leucine by reticulocytes with different concentrations of KCl in the suspension medium.

KCl present in Schulmann medium (mM)	Valinomycin (10^{-5} M)	(cpm)	Inhibition (%)
5	0	27000	
5	+	1500	94.3
28	+	1780	93.4
42	+	1950	92.6
70	+	2475	90.8
84	+	2500	90.5
98	+	2365	91.2

0.1 ml of reticulocytes were resuspended in 1 ml of Schulmann medium containing the indicated concentrations of KCl, after 40 min incubation at 37° the cells were counted for hot TCA precipitable material. A control of cells nontreated with valinomycin and suspended in the same medium was added for each KCl concentration.

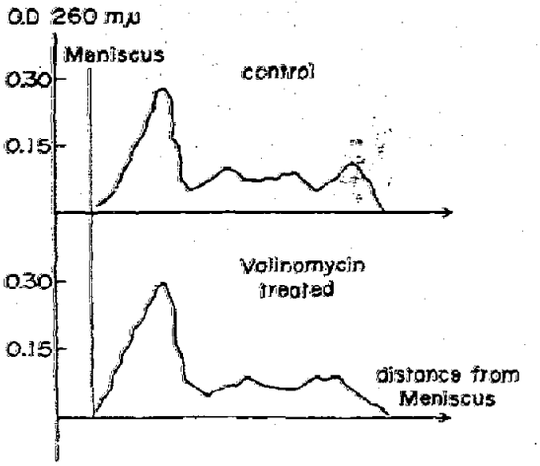

Fig. 2. Ribosomal fraction from Valinomycin-treated (lower curve) and control (upper curve) cells were run in a Model E ultracentrifuge and their profile of sedimentation analysed using a UV scanner. A typical pattern is shown here.

In order to see if this effect was caused only by a change in which the internal concentration of K^+ under the influence of the drug reached equilibrium with the external K^+ concentration, reticulocytes were incubated with or without valinomycin in Schulmann medium containing different concentrations of KCl (table 1). The results presented show clearly that valinomycin-induced inhibition of incorporation does not depend upon the KCl concentration in the incuba-

Table 2
Level of action of valinomycin on [^{14}C]leucine incorporation into polypeptides.

Supernatant fraction from:	Ribosomes from:	(cpm per 50 μg of ribosomes)
Untreated cells	Untreated cells	1900
Untreated cells	Valinomycin treated cells	470
Valinomycin treated cells	Valinomycin treated cells	440
Valinomycin treated cells	Untreated cells	1650

Supernatant and ribosomal fractions were isolated separately either from Valinomycin treated or control cells and a cell-free system was constituted with the different cross possibilities. The cell-free system was incubated for 20 min at 37° and counted for hot TCA precipitable material.

tion medium since such an antibiotic-induced effect remains unchanged in the presence of KCl concentrations that exist normally inside the reticulocytes. Moreover in a cell free system, the incorporation rate was not very sensitive to a lowering of the KCl concentration.

Furthermore, amino acid transport is relatively insensitive to those levels of Valinomycin used, since the uptake of [^{14}C]leucine was not reduced by more than 15% during the time of incubation. Such results could not account for the 90% decrease in incorporation in the presence of Valinomycin. Moreover, the ATP level, when measured by the luciferin–luciferase enzyme assay, remains at normal levels in the treated cells. It is, thus, unlikely that the inhibition of protein synthesis by the drug was due to a loss of energy. These two points were further controlled by using the cell free system. In order to test if another product having comparable external chemical structure and acting as an ionophore without being a polypeptide would have effects similar to Valinomycin, the cyclic polyether dicyclohexyl-18-crown 6 (D.C.) [10, 11] which has a marked affinity for K^+ was used. Indeed, an inhibition of protein synthesis was observed using D.C. but only when used at markedly higher concentration (fig. 1).

3.2. *Effect of Valinomycin on incorporation of [^{14}C]leucine in cell free system*

However unlikely, due to its strong binding to membranous structures, Valinomycin could inhibit protein synthesis by interacting directly with the ribosomes or the different enzymes necessary for the translation process. We tried to obtain an *in vitro* effect of Valinomycin in cell-free systems of incorporation and did not find any effect on the incorporation level, even at 10^{-4} M Valinomycin.

3.3. *Level of action of Valinomycin*

When ribosomes and S100 supernatant fractions were isolated from Valinomycin treated cells, the cell-free system failed to incorporate amino acids showing an inhibition of more than 60% as compared to control. However, when we used a cross complementation system in which the supernatant fraction from treated cells was added to ribosomal fraction from untreated cells, no change in the incorporation of the system was observed. However, when the supernatant fraction from cells were mixed with ribosomes from treated cells (table 2), an inhibition of incorporation comparable to that observed in the complete Valinomycin treated cell-free system was observed. These findings show that the inhibition is at the level of the ribosomal fraction and not of the supernatant fraction. This inactivation of the ribosomal fraction could not be reversed by simply adding KCl to the incubation mixture.

When studied by analytical ultracentrifuge, the profile of the ribosomal fraction from the treated cells was similar to the profile of the ribosomal fraction from untreated cells. Specifically, there was no quantitative difference in the level of 80 S ribosomes in the treated cells, showing that the inhibition was not due to stimulated RNAase activity (fig. 2). Morphologically, polysomes from the two fractions were identical when observed in the electron microscope. A run at low magnesium concentration which allows to differentiate between 59 S 'run off' ribosomes and 80 S 'RNAase' ribosomes (W. Bont, personal communication) showed that there were no more 'run off' ribosomes in the treated cells than in the controls.

Relief of the inhibition could be obtained by preincubating ribosomes from Valinomycin treated cells for 15 min with 0.5 M KCl. The percentage recovery obtained in different experiments was from 30% to 60% as compared to ribosomes from non-Valinomycin treated cells.

4. Discussion

It is known [12] that Valinomycin acts at very low dose level in the transport of K^+ by changing the resistance of the membrane to this cation in a very specific way [13–15]. The fact that we find an effect on a protein synthesis apparatus in spite of large fluctuations in the external K^+ concentrations shows that this effect is not due to the concentration changes. This is further corroborated by the fact that in a cell-free system which is not supplemented with external mRNA, the protein synthesis is not very sensitive to the KCl concentration in the medium.

That this effect of Valinomycin may be membrane mediated is shown by the fact that we did not find any inhibitory effect of the drug when acted on a cell-free system. This negates the possibility that Valinomycin as such could bind directly to the polysomes or to the factors and enzymes which are in the supernatant fraction.

The results shown in table 2 demonstrate clearly that the ribosomal fraction is the one which is inhibited when cells are treated with Valinomycin. However the fact that no more 59 S 'run off' ribosomes were found in the treated cells as compared to control cells, and the fact that the polysomal profile of the two fractions is identical, tend to show that this inhibition is neither at the level of RNAase nor of initiation. If RNAase activity was stimulated we would have found more 80 S ribosomes in treated cells than in control, and if initiation was stopped the amount of 59 S ribosomes would have been higher in treated cells and the amount of polysomes would have been lower as compared to control cells. It is then likely that the block is at the level either of termination or of elongation of the polypeptide chain. Which of the two possibilities is the right one is currently under investigation.

Kaufmann et al. found in a K^+ depleted mutant of *E. coli* that K^+ was necessary to maintain the peptidyl transferase reaction and that ribosomes isolated from this strain could be converted to an active form by preincubation in 0.1 M KCl or NH_4Cl [16]. Scheps et al. using ribosomes from *E. coli* at stationary phase found that they were blocked at the level of elongation and that this block could be relieved by incubation of the ribosomes either with 0.56 M KCl or Puromycin [17]. In our case the kind of inhibition induced by Valinomycin resembles more the one observed by Scheps et al. In mammalian cells Engelhardt [18] found a very similar effect on density inhibited cells.

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