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COUPLING BETWEEN K EFFLUX, ATP METABOLISM AND PROTEIN SYNTHESIS IN RETICULOCYTES

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SUMMARY

The relationship between three activities of the reticulocytes, potassium fluxes carried out by the cell membrane, ATP metabolism and protein synthesis are studied here. By comparing the three activities as a function of increased concentration of two potassium ionophores, valinomycin and dicyclohexyl-18-crown-6 (DC) the existence of coupling between these three activities was suggested.

We found two antagonistic effects of valinomycin on potassium efflux: (1) it inhibited potassium efflux at concentrations below 10nM; (2) it increased K efflux as expected, at concentrations above 20nM. Dicyclohexyl-18-crown-6, a macrocyclic polyether only reduced the membrane potassium efflux.

The two potassium ionophores, while inhibiting potassium efflux out of the reticulocytes, parallely inhibited protein synthesis. They also produced a parallel decrease in ATP/ADP ratio, which was small with valinomycin and much higher with dicyclohexyl-18-crown-6.

We were able to present here data suggesting that protein synthesis mechanism is coupled to the membrane activity, with the ATP metabolism as a probable intermediate.

INTRODUCTION

Evidence has been recently accumulated on the existence of coupling between the cell membrane activities and biosynthetic reactions in different biological systems (1-5).

A coupling between the potassium transport across reticulocyte membrane and protein synthesis within the cells has been suggested (6). It was shown that the two potassium ionophores, valinomycin and dicyclohexyl-18-crown-6 (DC) (7) inhibited protein synthesis in reticulocytes and this inhibition was not due to changes in the intracellular potassium concentration. In addition, it was shown that DC and low concentration of valinomycin acted reversibly and its inhibitory effect could be released by washing the reticulocytes. No effect was detected by adding the drugs to a reticulocyte cell free system, which suggested that the inhibition is likely to be membrane mediated (6). Valinomycin is a cyclic

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peptide antiobiotic that acts by greatly increasing the permeability of membranes specifically to potassium ions (8-11). Breitbart et al. (12) measured the effect of valinomycin on K influx across the reticulocyte membrane. They could not show any effect on K influx at valinomycin concentrations below 10^{-5}M even though the inhibition of protein synthesis started at much lower concentrations of the ionophore (6,12). However, K influx into the reticulocyte is composed of ouabain sensitive K influx and ouabain resistent K influx. Thus the possibility incited that a modification of the ouabain resistent K flux was probably masked by the ouabain sensitive one. Therefore we developed a sensitive assay which differentiates between the two fluxes by measuring K efflux in the presence and absence of ouabain from ^{42}K loaded red cells. Under these conditions we found that DC and low concentration of valinomycin reduce ouabain resistent K flux through the reticulocyte membrane. Under the same conditions the two K ionophores strongly inhibit protein synthesis in the reticulocytes. Moreover, evidence is given for a possible intermediate step in the coupling namely a subtle interference with ATP metabolism.

METHODS

Materials

^{42}K was obtained from the Israel Atomic Energy Commission of the Soreq Nuclear Research Centre. [^{14}C] leucine and [^{3}H] adenine were supplied by the Nuclear Research Centre, Negev, Israel. All the other chemicals used were of analytical standard. Valinomycin was purchased from Sigma and dicyclohexyl-18-crown-6 was a gift from Eli Lili Co. Reticulocytes were prepared and protein synthesis was measured as described by Freudenberg and Mager (13). The incubation medium contained 10µl reticulocytes in 0.5ml medium A (containing 155mM NaCl, 5mM KCl, 10mM glucose) and 0.1µCi ^{14}C leucine.

K efflux

10ml 10% reticulocyte or erythrocyte suspension in Na-Ringer were incubated in the presence of 10mM KCl and 1mCi ^{14}K at 37° for 2 hours, with gentle shaking, and the ^{42}K loaded cells were cooled, and washed 4 times with cold saline, the last wash being carried out with medium A. 50µl of ^{42}K loaded cells were transferred to medium A (2.5ml) containing 0.05 mg/ml ouabain at 37°. Samples of 0.5ml of each system were cooled and centrifuged for 3 min. at 3000 rpm. Radioactivity was counted on the supernatents. In order to eliminate errors as a result of ^{42}K decay the samples were counted for only 2 min, and an internal standard being set after every 3-5 samples. Valinomycin was added in ethanolic solution and the same amount of ethanol was added to the controls. Freshly dissolved ouabain was used.

Measuring the Intracellular adenine nucleotides

The intracellular nucleotides were labelled according to Freudenberg and Mager (13) The labelled cells were incubated with the K ionophores for 5 min. at 37° and the nucleotides were extracted and separated as described by Freudenberg and Mager (13).

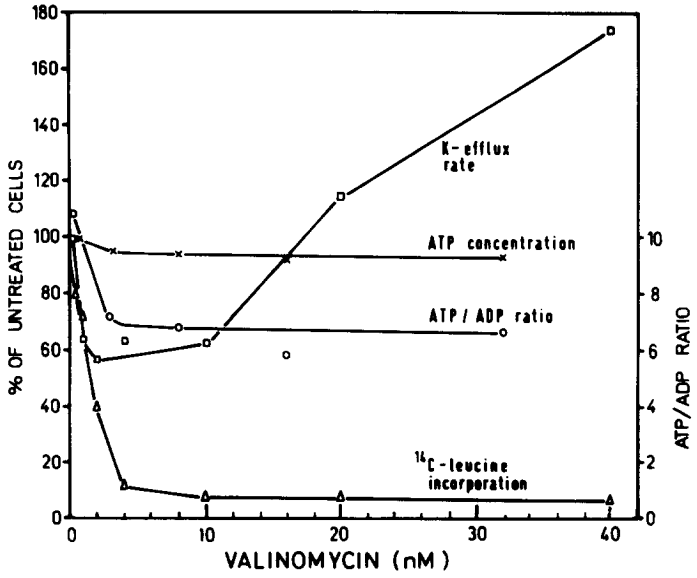

Figure 1: The effect of valinomycin on K efflux rate, ^{14}C leucine incorporation and ATP metabolism in rabbit reticulocytes
K efflux conditions, ^{14}C leucine incorporation and ATP concentration as described in methods.

RESULTS

Relationship between K fluxes and protein synthesis in reticulocytes

The possibility of a coupling between protein synthesis in the reticulocyte and K fluxes through its membrane arose after valinomycin and dicyclohexyl-18-crown-6 (DC) known as K ionophores were found to inhibit protein synthesis in reticulocytes (6). The results presented in Figure 1 and 2 show that more than 90% of protein synthesis activity was inhibited at concentrations of valinomycin and DC as low as 4nM and 0.05mM respectively. We found that parallel to protein synthesis inhibition the two K ionophores reduced K efflux out of the reticulocyte. The inhibitory effect of low concentration of valinomycin and of DC on K efflux was found only in reticulocytes and not in erythrocytes (not shown here). Moreover valinomycin above 20nM increased K efflux as expected whereas DC does not enhance the K efflux at concentrations up to 1.0mM (not shown here).

Figure 1 and 2 show the activities altered by valinomycin and DC, namely K efflux ^{14}C leucine incorporation into protein, ATP level and ATP/ADP ratio.

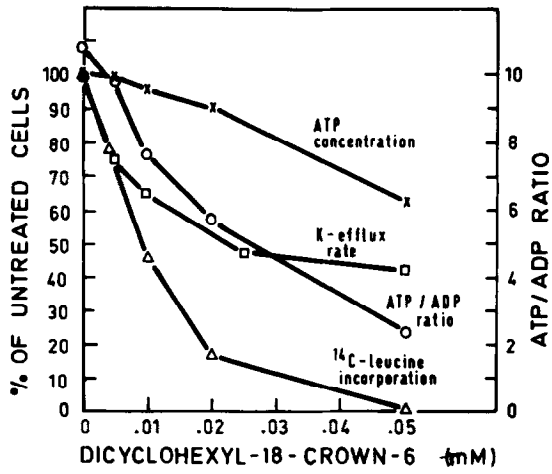

Figure 2: The effect of DC on K efflux, ^{14}C leucine incorporation and ATP metabolism in reticulocytes.
K efflux conditions, ^{14}C leucine incorporation and ATP concentration as described in methods.

The decrease in ouabain resistent K efflux by valinomycin and DC and the decrease in $[^{14}C]$ leucine incorporation into protein are parallel. Where protein synthesis inhibition by valinomycin (4nM) and DC (0.05mM) reaches its maximum, K efflux rate is minimum. This result suggests a coupling between protein synthesis and ouabain resistent K flux carried out by the cell membrane.

Valinomycin and dicyclohexyl-18-crown-6 effect on ATP metabolism

In order to determine whether the inhibition of protein synthesis by the two potassium ionophores is not merely the results of their action as uncouplers of oxidative phosphorylation or any effect on ATP metabolism, their effect on the adenine nucleotides pool was measured. Table I shows that even at valinomycin concentration of 20nM in the absence of glucose, the ATP concentration declined by almost 20% which could account for the high protein synthesis inhibition (13). However, in the presence of glucose at valinomycin concentrations up to 1μM the ATP was not decreased by more than 10%.

On the other hand, we found that DC as low as 0.05mM has a stronger effect on the ATP level than valinomycin has, even when glucose was added, which could be

TABLE I

Effect of valinomycin on the adenine nucleotides pool in presence and absence of glucose

Valinomycin concentration	with glucose in medium % of total adenine nucleotides			ATP/ADP	without glucose in medium % of total adenine nucleotides			ATP/ADP
nM	ATP	ADP	AMP		ATP	ADP	AMP	
0	87.9	8.2	1.2	10.7	87.8	9.6	0.9	9.1
4	83.5	11.7	2.5	7.1				
10	82.7	12.1	2.6	6.8				
20	80.5	13.9	2.6	5.8	69.7	16.6	7.6	4.2
40	81.6	12.4	3.8	6.6				
200	78.0	15.1	3.7	5.2	62.3	20.5	9.9	3.0
1000	83.5	11.9	2.4	7.0	68.1	17.2	5.9	4.0

Adenine nucleotides were labelled, separated and quantitied as described in methods.

as a result of its uncoupling effect on the oxidative phosphorylation of the reticulocytes (Table II).

Figure 2 shows that the DC concentration dependence curves are parallel for the three parameters: namely, K efflux,protein synthesis and ATP/ADP ratio. Valinomycin at concentration up to 10nM behaves similarly to DC. Where protein synthesis inhibition by valinomycin and DC reaches its maximum, the K efflux rate is minimum. On the other hand, the decrease in ATP at these points is only 5% with valinomycin (4nM) and 37% with DC (0.05mM). However, 0.02mM DC which inhibits 85% of protein synthesis activity and 50% of the K efflux, decreases only 10% of the ATP level. Therefore, it seems that protein synthesis inhibition by the two K ionophores is not simply as a result of ATP depletion. The ATP/ADP ratio could be a more sensitive control mechanism (13). If this small change in ATP concentration or in ATP/ADP ratio induced by valinomycin and low DC is responsible for the high inhibition of protein synthesis, it appears to be here an indication for a very sensitive control mechanism. It seems from these results that DC has a stronger effect on ATP metabolism than valinomycin. On the other hand, in vivo protein synthesis is inhibited similarly by these two K ionophores at concentrations which produce inhibitio of the K efflux.

TABLE II

The effect of DC on adenine nucleotides pool in reticulocytes in the presence of glucose

DC concentration mM	% of the total adenine nucleotides ATP	ADP	AMP	ATP/ADP
0	87.9	8.2	1.2	10.8
0.005	87.2	8.9	2.1	9.9
0.01	84.0	11.0	2.7	7.6
0.02	79.0	14.2	3.7	5.6
0.05	54.9	22.8	20.0	2.4

Adenine nucleotides were labelled, separated and quantitied as described under methods.

DISCUSSION

In this paper we are presenting results which suggest coupling between K fluxes across the reticulocyte cell membrane and protein synthesis. Furthermore, we suggest that ATP metabolism is an intermediate step in this coupling.

Two K ionophores, valinomycin and DC, were shown to inhibit K efflux out of reticulocy and at the same time to inhibit protein synthesis. These two K ionophores were previously shown to inhibit protein synthesis in rabbit reticulocytes but not in cell free extracts. This inhibition was almost instantaneous and reversible at DC concentration of up to 1mM and valinomycin of up to 10^{-8}M. The protein synthesis inhibition by the two K ionophores was observed under conditions that allowed for no change in K cell concentration, moreover depletion in amino acids was ruled out (6). Although results indicate that the inhibition of protein synthesis is related to the cell membrane, Breitbart et al (12) were unable to show any effect on K influx below 10^{-5}M valinomycin. By measuring K efflux directly and in the presence of ouabain, we could detect the unexpected inhibitory effect of DC and low concentration of valinomycin. Breitbart et al could not possibly see the inhibitory effect of the two K ionophores since they measured total K influx rather than ouabain resistent K efflux. Ouabain resistent K influx is only 10-20% of the total influx in rabbit reticulocytes. The ouabain sensitive influx is not influenced by K ionophores, therefore masked the ouabain resistent one. The maximum inhibition of K passive permeability by the two K ionophores is approximately 50% (Figure 1 and 2), thus

it was impossible to notice 50% inhibition of the 10% ouabain resistent flux without first inactivating the 90% ouabain sensitive flux.

The inhibition of K efflux out of the reticulocyte observed by low valinomycin and DC is unexpected since K concentration gradient across the membrane is in favour of K efflux. By adding K ionophore known to increase K passive permeability, one should expect an increase of K efflux out of the cells into surrounding medium (which contain low K (5mM) compared to the reticulocyte cytoplasm).

The protein synthesis activity is not simply inhibited by reducing K passive efflux. Under the condition that K efflux was reduced to near zero by reduction of its concentration difference to zero, we could not find any change in protein synthesis activity (not shown here).

This inhibitory effect of the two K ionophores on K efflux out of reticulocytes could be explained by a competition with the natural K carrier. Should one assume that the affinity (or apparent Km) of valinomycin and DC are higher than the affinity of the K carrier to K ions, but the diffusion coefficient of the complexes and/or their dissociation rate constant are lower. By competing with the carrier the K ionophores would bind to K ions better but would be transported and dissociated at a slower rate. This would account for the overall inhibition of the K efflux out of the reticulocyte.

Similar competition between valinomycin and various lipophilic anions for absorption sites at the membrane interface was reported by few groups (14,15). It was demonstrated that by adding lipophilic anions to bilayer membrane they block K conductance induced by valinomycin. We have now evidence for the existence of K carrier in reticulocyte cell membrane, which support the above theory on the mechanism of valinomycin inhibition of K efflux.

K efflux and protein synthesis activity are similarly inhibited by increasing concentrations of valinomycin (Figure 1) and DC (Figure 2). It has been suggested before that protein synthesis is inhibited by modification of ATP metabolism induced by various oxidative phosphorylation uncouplers (13). From their results it was clear that the important parameter was not so much the amount of ATP as the energy source.

The ATP/ADP ratio, as an indication on ATP metabolism (synthesis and breakdown) overall rate, is apparently the important parameter.

The effect of the two K ionophores on ATP metabolism was determined by measuring adenine nucleotides pool. We showed that valinomycin has a slight effect on ATP level, no more than 5% decrease in ATP has been found by valinomycin up to 1μM (Table The decrease in ATP and ATP/ADP ratio reaches a plateau above 4nM valinomycin where K efflux and protein synthesis at its minimal rate. On the other hand, DC has more pronounced effect on ATP/ADP ration, which could be as a result of its stronger uncou effect. The effect of DC and valinomycin on ATP level could be as a result of their action as oxidative phosphorylation uncouplers. However, intact mitochondria have nc been seen in rabbit reticulocytes, though indication on its activity were reported (1 The results presented in this paper may provide an indirect argument for the localizat of oxidative phosphorylation bound to reticulocyte cell membrane, for example in the form of mitochondria fragments bound or integrated in it.

In summary, our results suggest that a change in K efflux would cause a reductic in ATP/ADP ratio, and this in turn would inhibit protein synthesis. The mechanism of this coupling is very interesting and remains to be elucidated.

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