INHIBITORY EFFECTS OF TWO POTASSIUM IONOPHORES ON OUABAIN-RESISTANT POTASSIUM FLUXES IN RETICULOCYTE CELL MEMBRANE

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1. Introduction

Valinomycin is a well-known cyclic depsipeptide antibiotic that acts by greatly increasing the permeability of various biological membranes, specifically to K^+ [1–4]. A second compound which has the same kind of specificity for K^+ is dicyclohexyl-18-crown-6 (DC) which belongs to a family of synthetic ionophores [5]. Valinomycin was shown to increase K^+ efflux out of red cells at as low a concentration as 10^{-7} M [3]. In contradiction to these results on mature red cells, no effect of low valinomycin on K^+ permeability of the reticulocyte membrane was found [6]. However, they did not differentiate between passive and active influx, measuring only active influx assuming that it reflected K^+ efflux, through K^+:K^+ exchange. We developed a sensitive assay for measuring K^+ passive and active fluxes through the red cell membrane and were able to find unexpected inhibitory effects of two K^+ ionophores on the ouabain-resistant fluxes of K^+ through the reticulocyte cell membrane.

2. Materials and methods

$^{42}K^+$ was obtained from Israel Atomic Energy Commission of the Soreq Nuclear Research Centre, and $^{86}Rb^+$ from New England Nuclear. Valinomycin was purchased from Sigma and dicyclohexyl-18-crown-6 was a gift from Eli Lilly. Reticulocytes were prepared according to [7], washed twice with 5 vol. cold saline and suspended in Na^+-Ringer solution without Ca^{2+}.

2.1. $^{42}K^+$ *efflux*

2.1.1. Loading the cells with $^{42}K^+$

$^{42}K^+$ (1 mC) and KCl (10 mM) was added to 10 ml cell suspension (10%) then incubated at 37°C for 2 h under continuous gentle shaking. At the end of the incubation period the cells were cooled, washed 4 times with cold saline, finally washed with 155 mM NaCl, 5 mM KCl, 10 mM glucose (solution A) and suspended in it.

2.1.2. $^{42}K^+$ efflux

The reaction was started by adding 50 μl $^{42}K^+$ loaded cells to 2.5 ml solution A containing 0.05 mg ouabain/ml and incubating it at 37°C. At intervals, samples were centrifuged at 4°C for 3 min at 3000 rev./min. The $^{42}K^+$ efflux was measured by counting the radioactivity in the supernatants. The pellets were washed 3 times with 6 ml cold saline and hemolyzed in 1.0 ml water. The specific activity of $^{42}K^+$ was determined by counting radioactivity in the washed cell pellets and measuring total K^+ by Perkin Elmer Atomic absorbance spectrophotometer.

2.2. $^{42}K^+$ *influx*

The cells were treated as in the efflux assay with the exception that the 2 h incubation at 37°C was carried out without the radioactive K^+. Each system contained 155 mM NaCl, 5 mM KCl, 2 μCi $^{42}K^+$, 10 mM glucose and 0.05 mg/ml ouabain in 2.5 ml final vol. The reaction began by 50 μl cells, incubation was carried out at 37°C. At intervals, 0.5 ml samples were transferred to 4.5 ml cold saline and centrifuged at 4°C. The pellets were washed 3 times

with cold saline, hemolyzed in 1.0 ml water and counted. Specific activity of $^{42}K^+$ was determined by Perkin Elmer atomic absorbance spectrophotometer.

2.3. $^{86}Rb^+$ influx (as a tracer for K^+)

Each system contained: 310 μmol NaCl, 10 μmol RbCl 2 μCi $^{86}Rb^+$, 20 μmol glucose, 0.1 mg ouabain and 80 μl cells in 2 ml final vol. The reaction began with the addition of cells, incubation was carried out at 37°C; at intervals 0.5 ml samples were cooled, washed and counted as described in K^+ influx assay.

3. Results

Table 1 compares the K^+ fluxes in rabbit erythrocyte with those of the reticulocytes. As can be seen the ouabain-sensitive K^+ influx in reticulocytes is 7–8-times higher than the ouabain-sensitive K^+ influx in the red cells, indicating a higher activity of the Na^+–K^+ pump in the reticulocytes. Ouabain, known as a specific inhibitor of the Na^+–K^+ ATPase blocks 80–90% of the influx whereas the efflux is not inhibited (see table 1). Therefore, the K^+ efflux or K^+ influx in the presence of ouabain is mostly a function of K^+ passive permeability of the cell membrane, whereas K^+ influx without ouabain is mostly due to the activity of the Na^+–K^+ pump. As shown in table 1 the K^+ efflux and ouabain-resistant K^+ influx across reticulocyte membrane is much higher than these K^+ fluxes across the red cell membrane. This difference by itself indicates the existence of a mechanism for K^+ transport different in reticulocyte from that in the mature red cell.

Figure 1 compared the effect of valinomycin on K^+ efflux out of rabbit erythrocytes with its effect on

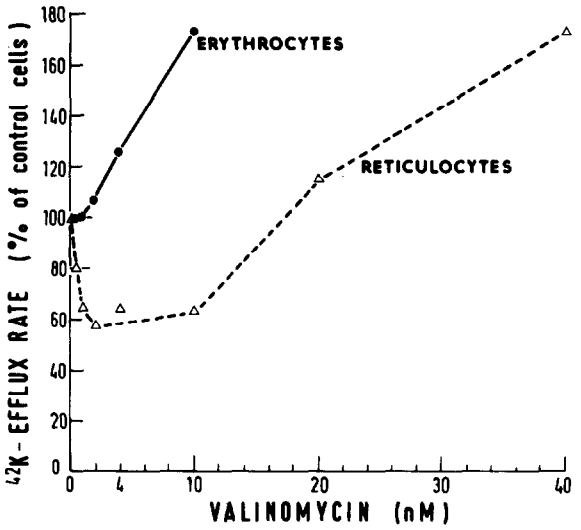

Fig.1. The effect of valinomycin on K^+ efflux from rabbit erythrocytes and reticulocytes. Valinomycin was added in ethanolic solution and the same amount of ethanol was added to the control. K^+ efflux conditions as described in section 2.1.

K^+ efflux out of reticulocytes. To our surprise we found two antagonistic effects on K^+ passive efflux out of the reticulocytes:

(1) At valinomycin < 10 nM, it inhibits K^+ efflux.
(2) At valinomycin > 20 nM, it increased K^+ efflux as expected.

This unexpected inhibitory effect of valinomycin was found only in reticulocytes and not in mature erythrocytes. In erythrocytes only the expected stimulation of the K^+ efflux was observed even with the low concentrations of valinomycin. By adding DC to reticulocytes it never enhanced K^+ efflux out of the reticulocytes (fig.2). Increasing DC concentra-

Table 1
K^+:K^+ exchange in rabbit erythrocytes and reticulocytes

Addition	Flux rate (mmol/l cell/h)			
	Red cells		Reticulocytes	
	Influx	Efflux	Influx	Efflux
–	4.2	6.6	30.0	25.2
Ouabain (0.05 mg/ml)	0.4	7.2	7.2	31.8

The K^+ efflux and K^+ influx conditions as described in section 2

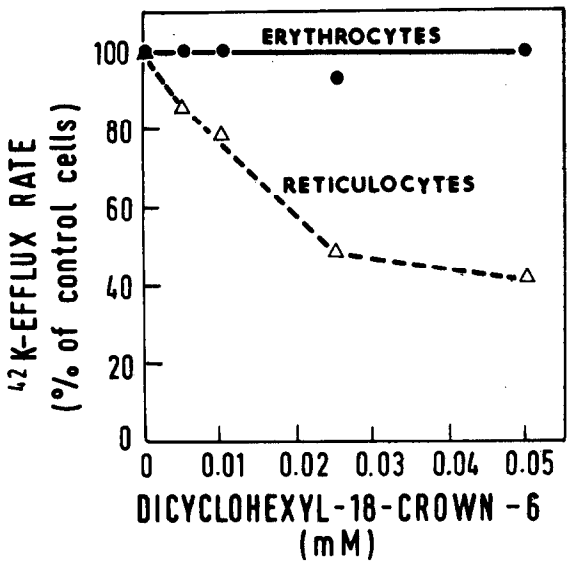

Fig.2. The effect of DC on K^+ efflux, from rabbit erythrocytes and reticulocytes. K^+ efflux conditions as described in section 2.1.

tions produced inhibition of the K^+ efflux out of reticulocytes, as do low concentrations of valinomycin. The inhibitory effect of the two K^+ ionophores (low concentrations of valinomycin and DC tested up to 1 mM) on K^+ efflux were found only in the reticulocytes and not in the erythrocytes. It was shown [4] that addition of H^+ conductors greatly increased valinomycin-promoted K^+ efflux by facilitating K^+ exchange with H^+ in erythrocytes. To test whether the different effects of the two K^+ ionophores on reticulocytes and erythrocytes K^+ efflux is not due to different permeability of their membrane to H^+, we compared the effect of H^+ conductor carbonylcyanide *m*-chlorophenylhydrazon (CCCP) in the two cells. Table 2 shows that the addition of 1.5 nM valinomycin induced 40% inhibition of Rb^+ influx in the reticulocytes and only a small inhibition in erythrocytes. By adding valinomycin in the presence of CCCP there is 6–7-times increase in ouabain-resistant Rb^+ influx in erythrocyte (in agreement with [4]) and no stimulation in reticulocyte and even to some degree of additional inhibition (table 2). This experiment indicates that the valinomycin-induced inhibition of K^+ fluxes through the reticulocyte membrane is not due to limited H^+ permeability.

4. Discussion

We showed here that ouabain-resistant K^+ fluxes are higher in the reticulocyte membrane than in the erythrocyte membrane. These high ouabain-resistant K^+ fluxes in the reticulocyte are reduced by adding two K^+ ionophores, valinomycin at low concentrations and DC. This unexpected inhibitory effect of the two ionophores on reticulocytes was proven not to be a result of limited H^+ permeability. It could be explained by a competition with a natural K^+ carrier in the reticulocyte membrane, by assuming that the affinities (or app. K_m) of valinomycin and DC are higher than the affinity of the K^+ natural carrier to sites for K^+ transport, but the diffusion coefficients of the complexes and/or their dissociation rate constants are lower. The inhibitory effect of CCCP by itself on Rb^+ influx (table 2) could also be a result of

Table 2
The effect of CCCP and valinomycin on ouabain-resistant Rb^+ influx in rabbit reticulocytes and erythrocytes

Addition	Ouabain-resistant Rb^+ influx (mmol/l cell/h)	
	Red cells	Reticulocyte
–	0.32	3.55
1.5 nM valinomycin	0.35	1.92
10 μM CCCP	0.25	1.04
1.5 nM valinomycin + 10 μM CCCP	1.95	1.26

Rb^+ influx as described in section 2.3

competition on sites with the K^+ carrier. Similar competition between valinomycin and various lipophilic anions for absorption sites at the membrane interface was reported by several groups [8,9]. It was demonstrated that adding lipophilic anions to bilayer membranes can block K^+ conductance induced by valinomycin. We have now evidence for the existence of a carrier-mediated ouabain-resistant transport of K^+, specifically inhibited by furosemide and ethacrynic acid [10] in reticulocyte cell membrane. This supports the above theory on the mechanism of valinomycin inhibition on K^+ efflux. In addition, erythrocytes seem to have lost this carrier in the process of maturation [10].

This can explain both the observed low K^+ permeability of the red cell compared to the reticulocyte membrane under similar normal conditions (table 1), and the lack of inhibitory effect of valinomycin and DC on K^+ fluxes in erythrocytes. High K^+ active transport was found in sheep reticulocytes compared to mature red cells [11]. Similarly we have also shown that rabbit reticulocyte membrane has higher K^+ active transport than erythrocyte (table 1).

It seems that the decrease in K^+ active transport from reticulocytes to erythrocyte follows a decrease in passive K^+ permeability, itself a result of elimination or inactivation of a natural K^+ carrier in the cell membrane during the process of maturation.

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