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EFFECTS OF VALINOMYCIN ON LYMPHOCYTES INDEPENDENT OF POTASSIUM PERMEABILITY

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10^{-7} M valinomycin affects human lymphocytes in the following manner: (1) it is non-toxic; (2) it inhibits mitogenesis; (3) it causes a reduction in cell ATP; and (4) it causes a marked increase in steady-state Na^+ exchange. However, it has a minimal effect on cell ion (K^+, Na^+, Ca^{2+}, Mg^{2+}) contents and no effect whatever on K^+ exchange. Neither the fast nor the slow fraction of steady-state K^+ exchange is affected by 10^{-7} M valinomycin. The various reported effects of valinomycin on lymphocyte functions cannot be assumed to be due to changes in plasma membrane K^+ permeability. The mechanism of the increase in steady-state Na^+ exchange, and whether or not it is related to inhibition of mitogenesis, are unsettled issues.

Introduction

Valinomycin, a neutral compound that enhances ion permeability of artificial membranes selectively for K^+ [1,2], has been reported to inhibit mitogenic transformation and anti-immunoglobulin-induced cap formation in human lymphocytes [3,4]. The authors interpreted these effects as due to an altered K^+ permeability of the plasma membrane, and valinomycin has been used to study the cell potential of lymphocytes on the assumption that it may allow rapid passive K^+ equilibration to occur [5–7]. However, Montecucco et al. [8] found that although in mouse spleen lymphocytes 10^{-7} M valinomycin inhibited anti-immunoglobulin-induced capping, it caused a marked loss of ATP.

We report here the effect of valinomycin on cell viability, ion contents, ATP and steady-state $^{42}K^+$ and $^{22}Na^+$ exchanges in human lymphocytes. In these studies we use 10^{-7} M valinomycin as a reference point since it inhibits mitogenesis but is non-toxic.

Methods

Cell separation, incubation, viability and water content. Lymphocytes were prepared from the peripheral blood of healthy young adults. Technique of cell separation from blood using ficoll-hypaque, composition of medium, conditions of sterile incubation at 37°C, methods of separating cells from the incubation medium without washing them, measurement of trapped space in cell pellets using ^{14}C-labelled polyethylene glycol, determination of cell counts and erythrosin B dye exclusion, and determination of water content by drying were all as described previously [9,10]. Incubation medium contained 5 mM K^+, 145 mM Na^+, 1.3 mM Ca^{2+}, 0.9 mM Mg^{2+}, 135 mM Cl^-, 0.34 mM HPO_4^{2-}, 1.3 mM $H_2PO_4^-$, 14.3 mM HCO_3^-, 5.6 mM glucose, and 10% fresh autologous serum, pH 7.4. Valinomycin was obtained from Sigma (lot 94C-0091).

Cell K^+, Na^+, Ca^{2+} and Mg^{2+}. Lymphocytes were incubated 24–48 h, cells packed at $6000 \times g$ in an 0.4 ml polyethylene microtube, the tip con-

taining the pellet severed and weighed, and ions extracted in 6 ml 0.1 M HCl at 22°C for 48 h, as described previously [9,10]. Ions were measured by atomic absorption spectroscopy again as described [9,10]. Standards were prepared from the chlorides dissolved in the same extraction medium. Corrections were made for a trapped space of 10% of wet pellet weight [10]. The normal Ca^{2+} level we find is comparable to that reported by Jensen and Rasmussen [11].

Cell ATP. Cells were counted and then separated from the incubation medium by pelleting at $360 \times g$. Pellets were resuspended in 1.5 ml of acetic acid (pH 7.75) containing 100 mM Tris-HCl and 1.0 mM EDTA. The suspension was boiled 10 min and the supernatant taken for assay of ATP by a luciferin-luciferase technique. Dessicated firefly tails (Sigma, St. Louis, MO) were finely ground in clean dry sand (Fisher, King and Prussia, PA) and extracted with 10 ml of 0.1 M arsenate with pH adjusted to 7.4 with 1 M H_2SO_4. Grinding followed by extraction was repeated a total of three times. Ten mg of tails were used, and the final volume of the pooled extracts was adjusted to 100 ml with the same arsenate solution. The extract was centrifuged at $6000 \times g$ for 5 min. The clear straw-colored supernatant was diluted to desired volume with the arsenate buffer. Fifteen ml of 0.154 M $MgSO_4$ was added to 100 ml of extract which was then allowed to stand 2 h at room temperature. Enzyme extracts were used at this time and were not stored. Standard ATP solutions were prepared from 0.1 mM ATP in the same Tris/EDTA/acetic acid buffer that was used to extract ATP from the cells. Assays were run in mini-scintillation vials containing 5 ml of the extracted enzyme. 0.5 ml of the sample (or standard) was added, time started, and the vial capped and swirled and placed in the scintillation counter. Samples or standards were counted 1 min each. Light output slowly declines over several hours; hence standards and samples were counted at equal times after mixing. ATP was expressed as nmol/10^8 cells.

Mitogenic transformation. Tritiated thymidine incorporation into DNA at 72 h was assayed as described previously [12], and concanavalin A (Miles-Yeda) was used at 10 μg/ml.

Isotopic steady-state Na^+ and K^+ exchanges. Cells were preequilibrated 24 h with and without valinomycin, during which they were loaded with $^{22}Na^+$ and $^{42}K^+$ and were then separated from the preloading medium by spinning through an oil-dibutylphthalate mixture. They were then transferred to non-labelled medium of identical composition, with and without valinomycin. Details of the technique of ion efflux were described previously [10,13]. Counts appearing in the medium are determined, and data are expressed as 1 − (specific activity of medium at time t/specific activity of medium at infinite time), the infinite specific activity taken as that of the suspension. Cells were never washed after preloading with isotope and the data are presented without correction for residual labelled medium carried over from the preloading step. This carryover appears as an instantaneously-exchanging fraction of ion efflux.

Results

Mitogenic transformation

The results in Table I confirm those reported by Daniele and Holian [3] using phytohemagglutinin and show that valinomycin (10^{-7} M) inhibits concanavalin A-induced transformation as well.

Viability, water, ATP and ion contents

Control and valinomycin-treated cells were incubated simultaneously and the time-independent (steady-state or equilibrium) parameters determined at 24 h are shown in Table II. 10^{-7} M valinomycin has no significant effect on recover-

TABLE I

MITOGENIC TRANSFORMATION

Data expressed as cpm [^{3}H]thymidine incorporated into DNA of 10^6 cells at 72 h.

Expt.	Additions	[^{3}H]Thymidine (cpm/10^6 cells)
1	None	482
	Valinomycin 10^{-7} M	175
	Valinomycin 10^{-6} M	108
2	Concanavalin A 10 μg/ml	43 779
	+ Valinomycin 10^{-7} M	3 801
	+ Valinomycin 10^{-6} M	2 346

TABLE II

EQUILIBRIUM PARAMETERS

Cells incubated 24 h with and without valinomycin 10^{-7} M. Means ± S.E. of 3–7 separate experiments.

	Control	Valinomycin
Cell count (cells/ml)	2.3 ± 0.4	2.4 ± 0.3
Dye exclusion (% of cells)	89.0 ± 3.7	83.8 ± 4.8
Water (% wet weight)	77.2 ± 1.0	75.9 ± 0.5
ATP (nmol/10^8 cells)	140 ± 51	78.3 ± 26
$K^+ + Na^+$ (mmol/kg)	170 ± 5.4	167 ± 3.3
K^+ (mmol/kg)	112 ± 7.6	101 ± 6.2
Na^+ (mmol/kg)	58.2 ± 4.4	66.3 ± 4.3
Ca^{2+} (mmol/kg)	3.8 ± 0.4	3.7 ± 0.8
Mg^{2+} (mmol/kg)	9.2 ± 0.4	9.4 ± 0.5

able cell counts, dye exclusion, or cell water, and hence is not toxic to the cells. The normal ATP level in current experiments is 75–100 nmol/10^8 cells, or about 3 – 4 mM. This is similar to that reported by others in lymphocytes using a boiling extraction as we did [14] or a perchloric acid extraction [11,15,16]. In the experiments in Table II the large standard errors reflect the influence of two earlier experiments in which we encountered greater variation in absolute levels. However, the effect of valinomycin is consistent whenever compared to simultaneous controls, and it causes a reduction in the ATP level by an average of 35%.

Valinomycin (10^{-7} M) had a minimal effect on cell ions (Table II), causing a slight but insignificant decrease in K^+ and increase in Na^+, and no change in Ca^{2+} or Mg^{2+}.

Steady-state Na^+ and K^+ exchanges

Simultaneous steady-state Na^+ and K^+ exchanges in cells incubated 24 h with and without valinomycin were measured by an efflux technique. The results are shown in Fig. 1. 10^{-7} M valinomycin has no effect on K^+ exchange (Fig. 1a) and 10^{-6} M valinomycin seems to cause a slight increase in K^+ exchange (Fig. 1b). There was no change in the cell K^+ contents at either concentration, as manifest by the same extrapolations of the slower exponential fractions to the ordinate. In contrast, valinomycin at either concentration caused a marked increase in steady-state Na^+ exchange.

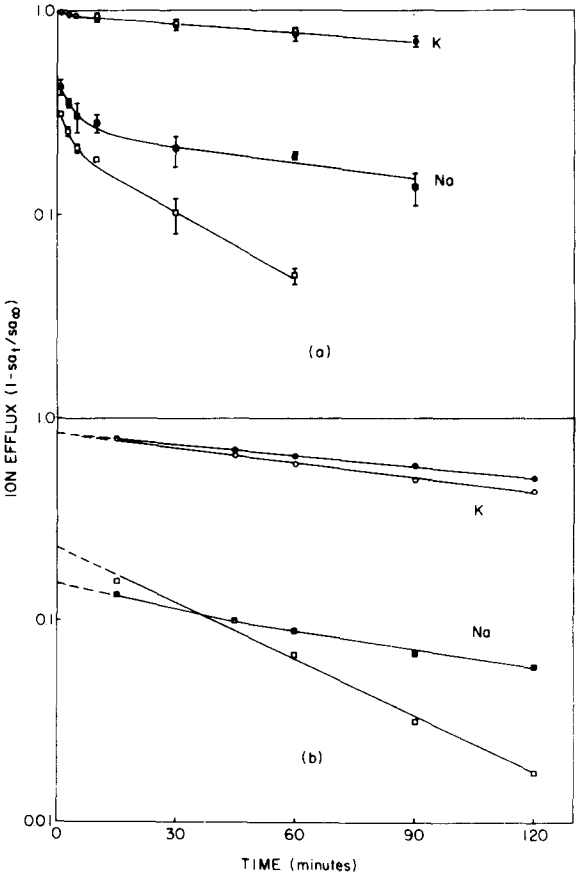

Fig. 1. (a) Simultaneous steady-state $^{42}K^+$ and $^{22}Na^+$ effluxes. Means and ranges of two separate experiments. ●, ■, Controls; ○, □, 10^{-7} M valinomycin. $^{42}K^+$ efflux was done in three additional studies with identical results. (b) The same with valinomycin at 10^{-6} M. ●, ■, Control; ○, □, 10^{-6} M valinomycin.

A study comparing early and late effects of valinomycin, in this case in cells in 32 mM K^+, is shown in Fig. 2. At 24 h, K^+ efflux is identical to that determined previously in control cells. At 5 min, K^+ efflux is actually slower than normal.

Effect of valinomycin on the fast fractions of K^+ exchange

It may be noted in Figs. 1 and 2 that the dominant exponential fractions of steady-state ion exchange are preceded by faster fractions. The different relative sizes of the fast and slow fractions of K^+ as compared to those of Na^+ reflect different relative amounts of ion exchanging in them. We have analyzed in depth the fast and slow

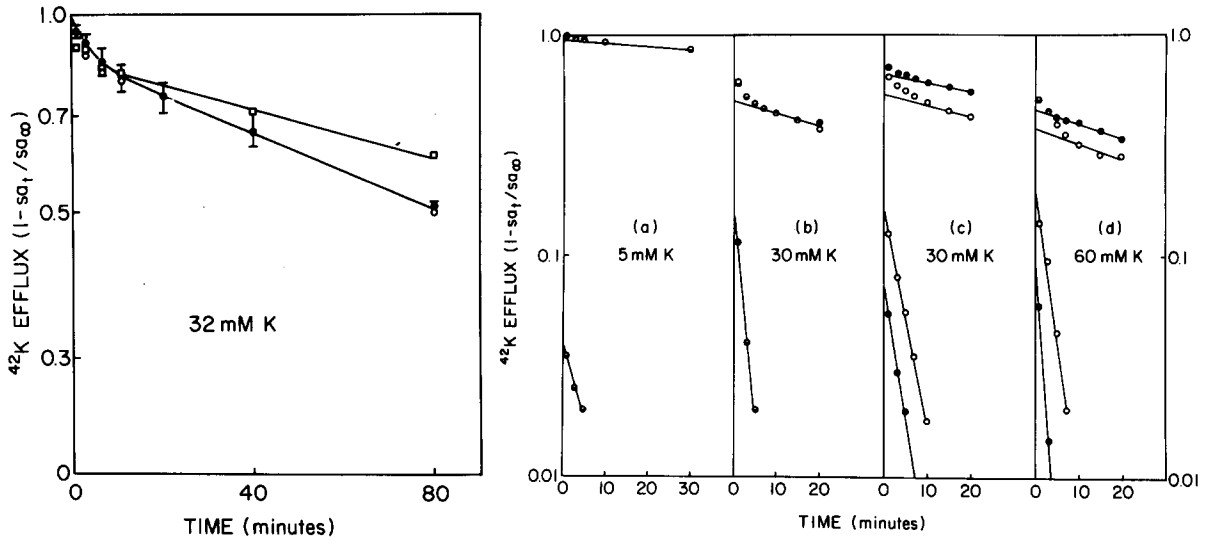

Fig. 2. Steady-state ^{42}K efflux at 32 mM external K^+ (118 mM Na^+) after 24 h. ●, Prior control cells (from Ref. 13); ○, 10^{-7} M valinomycin for 24 h; □, 10^{-7} M valinomycin for 5 min.

Fig. 3. Fast fractions of steady-state ^{42}K efflux. Cells preequilibrated 24 h at external K^+ concentrations indicated (external $K^+ + Na^+ = 150$ mM). ●, Control; ○, 10^{-7} M valinomycin.

fractions of K^+ and Na^+ exchange in human lymphocytes [10,13] and have found that 3% of normal cell K^+ and 50% of normal cell Na^+ exchange rapidly. We provided strong evidence that both fractions are cellular in origin and occur in series with one another, and suggested that the fast fractions may actually reflect the permeability of the surface plasma membrane. Therefore we attempted to define any possible effect of valinomycin on the rates of the fast fractions.

The data in Figs. 1 and 2 show that steady-state K^+ exchange reaches its slower dominant exponential fraction within 10 min. Steady-state K^+ exchange was followed for 20–30 min in the experiments shown in Fig. 3, and extrapolated backward from points at 10 min and later to obtain the size of the slower fraction. This was then subtracted from the total to yield the fast fraction. Within error, the fast fractions appear also to follow a single exponential function, and have half-times of exchange of about 2 min. These would correspond to a diffusion coefficient across the surface on the order of 10^{-10} cm^2/s (see Ref. 10), and the fast fractions have been shown not to be due to any sort of extracellular source or to surface binding sites (see Refs. 10 and 13). Analysis of the fast fractions is facilitated in cells equilibrated at higher external K^+, since the amount of K^+ in them increases while the amount of K^+ in the slow fraction remains constant [13]. The results in Fig. 3 show clearly that neither the fast nor the slow fractions of K^+ exchange are affected by valinomycin.

Discussion

These results show that a non-toxic concentration of valinomycin (10^{-7} M) has a marked effect on human lymphocytes in its inhibition of mitogenesis, its reduction of ATP, and its induction of an increase in steady-state Na^+ exchange. However, it causes slight or no change in cell Na^+, K^+, Ca^{2+} or Mg^{2+} levels, and it has no effect at all on the rate of steady-state K^+ exchange. Within the context of the membrane theory, one would have expected that in the steady-state any increase in K^+ permeability would have been manifested, even if it were balanced by an increase in K^+ pumping to prevent a net loss of cell K^+. We conclude that the marked effect of valinomycin on

various human lymphocyte functions is not due to an altered K^+ permeability.

Possible reasons for failure of valinomycin to increase K^+ exchange

Failure to see a net loss of K^+ in valinomycin-treated red blood cells has under certain conditions been attributed to an increased permeability to K^+ that leads to an increased membrane potential that in turn retards further K^+ loss. In order for appreciable net movement of K^+ to occur electroneutrality has to be maintained, either by permeant cations (such as H^+ or Na^+) moving in the opposite direction [17,18], or by permeant anions moving in the same direction [19,20]. However, the experiments describing these effects were in the absence of external K^+, while in the presence of external K^+, $^{42}K^+/K^+$ exchange occurred readily. Since in our experiments the medium contained normal (Fig. 1) or increased (Fig. 2) K^+, and since increased $^{42}K^+/K^+$ exchange in the steady-state did not occur, these phenomena could not account for our results.

Another possible reason for failure to see a net loss of K^+ is that a secondary increase in the pump (the (Na^+, K^+)-ATPase) compensates for an increased K^+ permeability. This, however, should have been manifested by an increased steady-state K^+ exchange.

Failure to see any increased K^+ exchange at all could be due simply to an insufficient concentration of valinomycin, its inactivation in solution, or other trivial factors. However, the inhibition of mitogenesis at 72 h, the effect on cell ATP, and the marked increase in Na^+ exchange indicate clearly that the valinomycin is quite active.

A final possible reason for failure to see an effect of valinomycin on K^+ exchange is that the normal K^+ permeability is already very high. This was considered as an explanation for failure of excitable membranes to respond to K^+ ionophores, but rejected on the basis of a variety of observations [21–25]. This is also not the case in human lymphocytes since K^+ loss is readily increased by incubation at low levels of external K^+ [13] or by treatment with lectins [26].

Comments on uses of valinomycin in lymphocytes

Valinomycin has been applied to lymphocytes with the assumption that it causes a selective increase in K^+ transport across biologic membranes. One use of valinomycin is to study the cell potential by allowing rapid passive K^+ equilibration to occur at various external K^+ concentrations in order to calibrate indirect measures of the potential, such as with fluorescent dyes. This technique yielded a null point (absence of fluoresence change with valinomycin) at 120 mM external K^+ in rat thymocytes, and hence a predicted potential of around 10 mV [5], but a null point at 12–15 mM K^+ in mouse spleen lymphocytes, and hence a potential of about 60 mV [7]. In human lymphocytes potentials of 30–60 mV were calculated from cell to medium K^+ ratios using lipophilic ion distribution in cells incubated at K^+ levels between 34 and 116 mM [6]. This would imply a potential of 80–90 mV in normal (5 mM) K^+. Similar results occurred in presence or absence of valinomycin. The potentials calculated from indirect estimates may be contrasted with those determined by direct microelectrode recordings in lymphocytes, which are consistently about 10 mV [27–29].

Our data do not address the question of the actual magnitude of the lymphocyte surface potential. However, they urge caution in assuming that the normal potential may be revealed by K^+ distribution in the presence of valinomycin. Bramhall et al. [5] did find a marked loss of $^{42}K^+$ from rat thymocytes; however, the concentration of valinomycin used, 10^{-5} M, may be toxic; the cells were preloaded with $^{42}K^+$ for only 50 min and then washed three times so that the fraction of total cell K^+ labelled with ^{42}K may have been small; the $^{42}K^+$ loss may not reflect net K^+ concentration changes; and as shown by Montecucco, Rink et al. [7,16] the high concentration of the fluorescent dye used may also be toxic.

The ability of valinomycin to inhibit mitogenic transformation and anti-immunoglobulin-induced cap formation in human lymphocytes [3,4] were overcome by increased external K^+ and were assumed to be due to an effect of valinomycin on the electrical properties of the cell membrane mediated by a change in the K^+ permeability. Montecucco et al. [8] however, reported in mouse spleen cells that although 10^{-7} M valinomycin inhibited capping, this effect was not overcome by increased

external K^+ and valinomycin caused a marked loss of cell ATP. For these and other reasons they felt that ATP depletion and not a membrane effect accounted for the inhibition of capping.

Our experiments in human lymphocytes support the general conclusion of Montecucco et al. [8] that valinomycin may have marked effects not due to cell surface membrane properties. However, our data suggest that the inhibition of mitogenic transformation [3] and of immunoglobulin capping [4] in human lymphocytes by 10^{-7} M valinomycin is not due to metabolic inhibition, since the decrease in ATP we observed did not coincide with any decrease in cell viability, any cell swelling, or any significant change in cell ion levels (Table II). We suspect that valinomycin may affect some cellular functions by mechanisms involving neither plasma membrane K^+ transport nor mitochondrial processes.

Effect of valinomycin on steady-state Na^+ exchange

In Ehrlich ascites tumor cells Levinson [30] found that valinomycin inhibited recovery of K^+ accumulation and Na^+ exclusion by cells depleted of K^+ and loaded with Na^+. Since this occurred along with loss of ATP, he concluded that absence of ATP for ion pumping explained the results. Poole et al. [31] found that 10^{-5} M valinomycin caused K^+ loss but also attributed this to ATP depletion. Smith and Levinson [32] documented a near-complete loss of ATP within 5 min; K^+ permeability, calculated from the net decrease in cell K^+ in presence of ouabain, increased by only 30%, and there was no change in the cell potential. They therefore considered the possibilities of increased Na^+ permeability or of stimulation of 'electrogenic' Na^+/K^+ active transport.

An increase of permeability selective for Na^+ would be unexpected from the known properties of valinomycin [1,2]. A secondary or primary increase in Na^+ pumping by the (Na^+, K^+)-ATPase should affect K^+ exchange as well; in fact, lymphocyte physiologists have used K^+ exchanges in order to assess functions of the Na^+ pump [14,26]. In the context of the membrane theory, the increased steady-state Na^+ exchange occurring in the absence of an increased steady-state K^+ exchange, would be considered most likely to reflect activation of an Na^+/Na^+ exchange diffusion mechanism [33]. In view of our prior observations in human lymphocytes [9,10,13,34–36], however, we suggest that the increased steady-state Na^+ exchange in the dominant slower exponential fraction reflects a decreased activation energy of Na^+-site interaction of sodium ions adsorbed onto intracellular macromolecules. Whether or not this effect on Na^+ is related to the inhibition of mitogenesis by valinomycin remains to be seen.

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