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A potassium ionophore (valinomycin) inhibits lymphocyte proliferation by its effects on the cell membrane

(inhibition lymphocyte mitogenesis/cations)

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ABSTRACT **Valinomycin is a depsipeptide antibiotic which selectively translocates potassium across biologic membranes. This potassium ionophore was observed to inhibit phytohemagglutinin-stimulated blastogenesis and proliferation in human lymphocytes. The effect was not due to toxicity to the cells, nor appeared to be due to the effects of valinomycin as an uncoupler of oxidative phosphorylation. Furthermore, the inhibitory effect on phytohemagglutinin stimulated lymphocytes was prevented by increasing the potassium concentration of the external media.**

These results suggest that the interaction of mitogens with specific receptors at the cell membrane may involve mechanisms affecting cation fluxes and membrane potential. These ionic events may play a role in the transduction of membrane signals for lymphocyte stimulation.

Since the initial observation by Nowell that phytohemagglutinin (PHA) stimulates lymphocytes to undergo blastogenesis and cell proliferation, (1) a number of other lectins, such as concanavalin A and pokeweed mitogen, have been shown to have similar mitogenic effects on lymphocytes (2, 3). These agents are thought to interact with membrane receptors and act as triggers for cell proliferation through a series of as yet ill-defined steps. More recently, the calcium ionophore (A23187) was also shown to induce lymphocyte proliferation (4). It has been suggested that A23187 initiates mitogenesis by translocating Ca^{++} across the membrane directly into the cytosol, thus bypassing events at the membrane.

We now present preliminary evidence that a potassium ionophore, valinomycin, can inhibit mitogenic stimulation by phytohemagglutinin. This effect was blocked by increasing the potassium concentration of the suspending media which suggests that mechanisms resulting in changes in membrane potential and ion fluxes at the cell membrane may play a role in lymphocyte stimulation by mitogens.

MATERIALS AND METHODS

Peripheral venous blood was obtained from healthy donors and collected in heparin. A lymphocyte preparation was obtained by gradient centrifugation using the Ficoll-Hypaque technique (5). Cultures were prepared by adding 5×10^5 cells to a 1.0 ml solution containing minimal Eagle's medium (MEM) plus pooled human AB serum (10% vol/vol) and 50 μg/ml of PHA-M (Difco, Detroit, Mich.). Cultures were incubated at 37° with 5% CO_2 in air for 72 hr. For studies of cellular proliferation, 0.25 μCi of [^{3}H]thymidine (specific activity 6.7 Ci/mM, New England Nuclear Corp., Boston, Mass.) were added to cultures 16 hr before termination of the culture. Cellular incorporation of [^{3}H]thymidine was determined by scintillation counting (6).

Valinomycin (Sigma, St. Louis, Mo., lot no. 94C-0091) was added to lymphocyte cultures prior to PHA and was present throughout the culture period. Valinomycin was dissolved in absolute ethanol; the concentration of ethanol to which cells were exposed did not exceed 0.1%. Viability and cell survival after exposure to all concentrations of valinomycin was tested by the trypan blue exclusion test and by cell counting (Coulter) using a modification of the cetrimid technique (7). Viability was always greater than 95%.

In certain experiments, the effects of monovalent cations were tested with lymphocytes cultured in MEM supplemented with KCl; in other experiments, equimolar concentrations of KCl replaced NaCl in the culture medium. In all experiments described below, the external medium contained 1.3 mM Ca^{2+} and 1.0 mM Mg^{2+}, 0.3 mM HPO_4^{-2}, 1.3 mM $H_2PO_4^-$, 1.4 mM HCO_3^-, and 5.6 mM glucose; the pH was maintained between 7.2 and 7.4. The potassium concentration of the final solution was determined by flame photometry. Osmolality of the solutions was determined in a milliosmometer.

In separate experiments, two uncouplers of oxidative phosphorylation, pentachlorophenol (Sigma, St. Louis, Mo.) (PCP) and carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) were tested in various concentrations for their effects on the proliferative response of lymphocytes to PHA. Both agents were dissolved in absolute ethanol; again, the ethanol concentration to which cells were exposed did not exceed 0.1%. Where specified, both uncouplers were added prior to the addition of PHA and were present throughout the culture period.

RESULTS

Inhibitory effects of valinomycin

The results of seven experiments indicating the effect of various concentrations of valinomycin (0.1 μM to 1 nM) on PHA-stimulated lymphocyte proliferation measured at 72 hr are shown in Fig. 1. At a valinomycin concentration of 0.1 μM, there was complete inhibition of the proliferative responses. At concentrations of 50 nM to 10 nM, there was partial inhibition with proliferative responses less than or equal to 20% of control values. Between 10 nM and 5 nM valinomycin, there was an abrupt decrease in the inhibitory effects of valinomycin. No significant effect was observed at valinomycin concentrations of 1 nM or less.

Inhibition of lymphocyte proliferation by valinomycin did not appear to be due to toxicity resulting in impaired viability. The viability of cells exposed to 0.1 μM to 10 nM valinomycin (without PHA) for up to 120 hr was within ± 15% of control lymphocyte cultures (without PHA or valinomycin).

Abbreviations: PHA, phytohemagglutinin; MEM, minimal Eagle's medium; PCP, pentachlorophenol; FCCP, carbonylcyanide *p*-trifluoromethoxylphenylhydrazone.

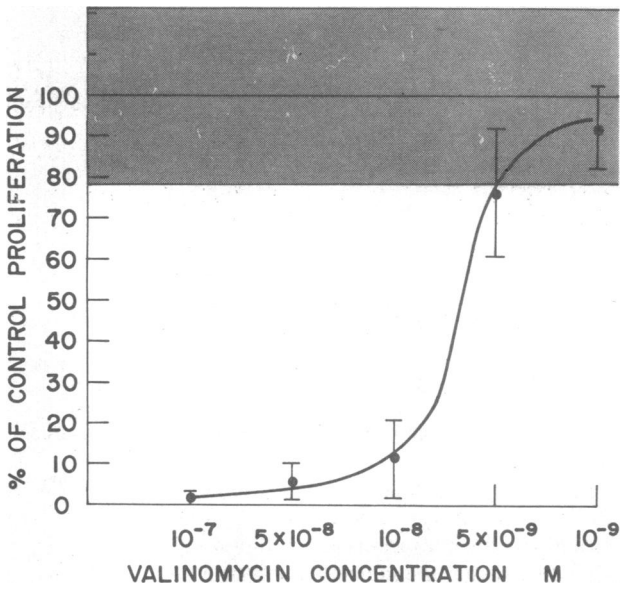

FIG. 1. Effect of various concentrations of valinomycin on the inhibition of lymphocyte mitogenesis measured by the incorporation of [^{3}H]thymidine at 72 hr. [^{3}H]Thymidine incorporation for control cultures (100%) equalled 65,500 ± 13,700. One standard deviation is shown by the shaded areas. Number of experiments = 10. • represents mean ± SD for seven experiments.

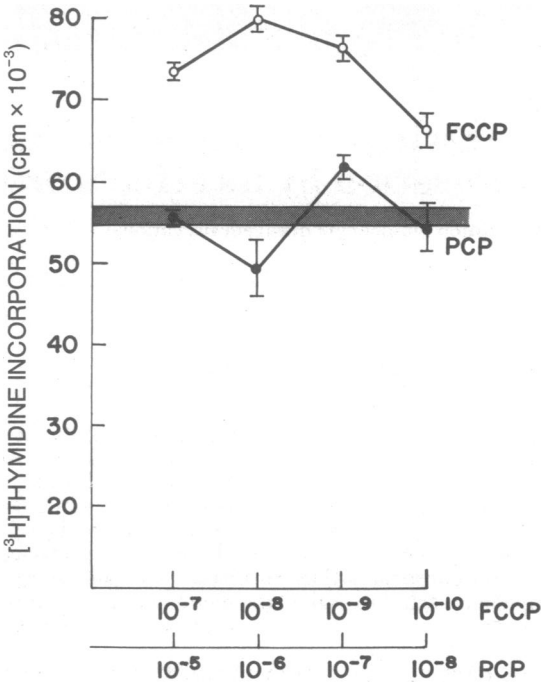

FIG. 2. Effect of uncouplers of oxidative phosphorylation (PCP, FCCP) on [^{3}H]thymidine incorporation in PHA stimulated lymphocytes measured at 72 hr.

Effect of uncouplers of oxidative phosphorylation

The inhibitory effect of valinomycin could be due to its effect as a mitochondrial inhibitor (uncoupler), although preliminary studies indicate that at extracellular concentrations of 10 nM or less, valinomycin does not uncouple oxidative phosphorylation. To determine what effect uncouplers of oxidative phosphorylation might have on lymphocyte mitogenesis, two relatively lipid soluble uncouplers, PCP and FCCP, were added to the lymphocyte cultures with PHA. As shown in Fig. 2, PCP had no significant inhibitory effect on lymphocyte proliferation over a wide range of concentrations (10 μM to 10 nM). Furthermore, although FCCP at 0.1 μM showed slight uncoupling of respiration, there was an increase in lymphocyte responses to PHA; FCCP increased lymphocyte stimulation by PHA as compared to controls at all concentrations tested (Fig. 2); and at 10 nM FCCP, lymphocyte proliferation was increased to 40% above control values. [^{3}H]Thymidine incorporation for cultures containing FCCP and PCP (no PHA) were equivalent to control cultures (no PHA or uncouplers). These results indicate that some uncoupling of mitochondrial oxidative phosphorylation may not inhibit lymphocyte blastogenesis by PHA and are consistent with the notion that the energy requirements for lymphocyte transformation may not be exclusively dependent upon ATP generated by mitochondria (8).

Effect of external potassium on the inhibitory effects of valinomycin

Evidence suggesting that valinomycin might exert its effect at the cell membrane rather than uncoupling of mitochondria was provided by increasing the potassium concentration of the external medium. As shown in Fig. 3, the proliferative response of lymphocytes cultured in the presence of 10 nM valinomycin and 5 mM of potassium was less than 1% of control. Increasing the potassium concentration of the external medium to 35–50 mM brought the proliferative response of valinomycin-treated lymphocytes to the level of control cultures. Increasing the potassium concentration beyond this level did not result in further enhancement of proliferation. Similar results were observed at 25 nM of valinomycin, but at 50 nM only slight (about 10%) recovery of lymphocyte response was observed at a concentration of 35 mM K^+ in the external medium; and, at 0.1 M, no proliferation was detected at any concentration of K^+ tested.

Effect of osmolality of the external medium

The osmolality of medium supplemented with 50 mM of KCl equaled 371 milliosmoles, an increase of about 100 milliosmoles

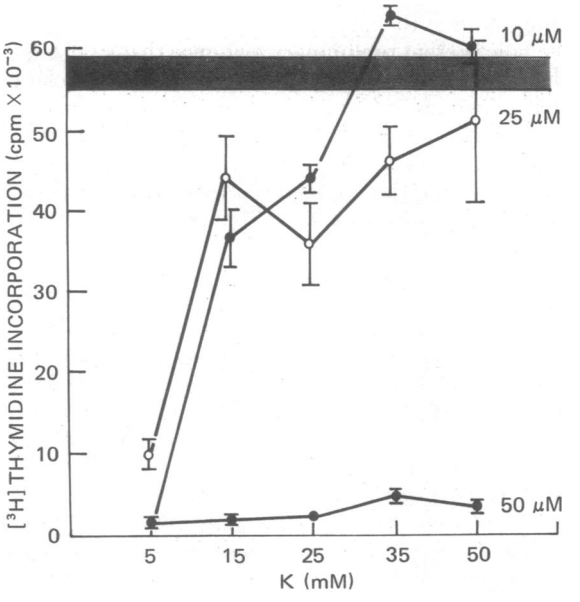

FIG. 3. Effect of increasing the K^+ concentration of external medium on [^{3}H]thymidine incorporation at 72 hr for lymphocytes cultured in the presence of three concentrations of valinomycin (50 nM, 25 nM, and 10 nM). Each point equals mean ± SEM of triplicate cultures of a representative experiment.

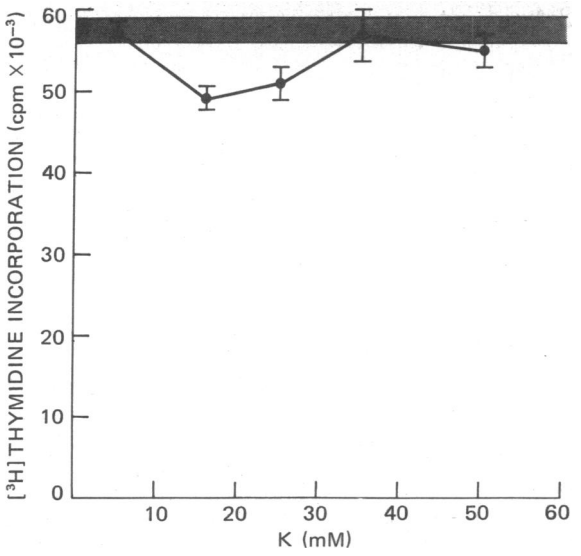

FIG. 4. [3H]Thymidine incorporation at 72 hr of PHA-stimulated lymphocytes in which KCl was added to culture medium.

over standard cultures. At this concentration of K^+, in the absence of valinomycin, there was no decrease in cell viability, nor a significant reduction in the proliferative response as compared to controls with standard levels of K^+ and osmolality (Fig. 4).

It is noteworthy that when NaCl was replaced by equimolar concentrations of KCl over a broad range (5–70 mM), instead of adding KCl to MEM, the inhibitory effect of valinomycin could not be consistently reversed (data not shown).

DISCUSSION

Valinomycin is a cyclic depsipeptide (molecular weight 1111.4) which forms lipid soluble complexes with monovalent cations (9–11). The ionophore is highly specific for potassium and creates an insulating cage (9, 10) around the ion allowing its transport across the lipid phase of both artificial and biological membranes. In the intact cell, it seems most probable that valinomycin exerts its effects at two sites at least: by energy uncoupling in the mitochondria (12–14), or by its action on the electrical properties of the cell membrane (15–17).

The present results, both with valinomycin and in preliminary studies with known uncouplers, suggest that the inhibitory effects of valinomycin on lymphocyte proliferation which we have described are not due to decreased cell viability, nor to its action as an uncoupler of oxidative phosphorylation; in our hands, 10 nM (or less) valinomycin, which reduced proliferation, did not affect mitochondrial uncoupling, and 0.1 μM FCCP caused partial uncoupling without impairing lymphocyte responses to PHA.

On the other hand, our observation that increases in extracellular K^+ did not reverse valinomycin at 0.1 μM or greater might imply that deleterious effects on intracellular membranes, including mitochondria and even the nucleus (18, 19) may become significant if extracellular concentrations of valinomycin are sufficiently high.

Clearly, the problem requires further study, and reports by others are conflicting. Several workers indicate that mitochondrial uncouplers, including potassium cyanide (20, 21) and 2,3-dinitrophenol (8) do not inhibit lymphocyte responses to PHA, but other investigators have stated that these same agents (22) as well as oligomycin and valinomycin (23) do abolish or inhibit PHA induced proliferation.

It seems probable, therefore, that valinomycin at the concentrations used in our studies exerts its effect at the cell membrane perhaps by altering electrical properties. In excitable tissues, where valinomycin has been more extensively studied, it selectively translocates potassium across the cell membrane. The increase in the K^+ permeability (Pk) may result in hyperpolarization or an impairment of the electrical behavior of the cell membrane (15–17). Application of the constant field equation (24, 25) predicts that, under certain circumstances, such a change in the membrane potential could be reversed by increasing the potassium concentration of the external medium (26). This prediction has been confirmed experimentally in both excitable (27) cells where the potassium permeability (Pk) is already relatively large, and in nonexcitable cells, where the potassium permeability is increased by valinomycin (26). Alternatively, valinomycin may act by exchanging intracellular K^+ for extracellular Na^+ or H^+. However, without a more precise knowledge of the relative concentrations and permeabilities of sodium and potassium, as well as chloride, in the lymphocyte, and the relative contribution to membrane potential made by electrogenic pumps (i.e., Na^+, K^+-ATPase), the present results can be considered only preliminary and indirect evidence implicating membrane potential and ionic exchange in the events leading to lymphocyte proliferation.

There is some further evidence bearing on the hypothesis that ion concentrations and fluxes are of importance in lymphocyte activation and proliferation in recent studies involving both lymphocytes and other culture systems: (*i*) using a microelectrode technique, Taki (28) observed a decrease in the resting membrane potential of small lymphocytes within minutes after the addition of PHA to the cell culture medium. However, others (29) have encountered difficulty in measuring stable membrane potentials in nonstimulated (PHA) lymphocytes due to their small size and scanty cytoplasm. (*ii*) Ouabain, which inhibits the membrane Na^+, K^+-ATPase electrogenic pump, reversibly abolishes the proliferative response to PHA (30). Also, PHA has been observed to increase the ouabain sensitive Na^+, K^+-ATPase activity in human and sheep lymphocytes (31). (*iii*) Lidocaine, which can decrease membrane cation conductance (32), also inhibits proliferative responses of lymphocytes to mitogens (33). (*iv*) In certain cell culture systems, contact inhibition of cell division is associated with hyperpolarization of the cell membrane (34). (*v*) Concanavalin A results in a marked increase in membrane conductance when added to bilayers prepared from sheep red cell lipids and a glycoprotein from human red cells (35). (*vi*) Additional studies indicate that concanavalin A also alters the lymphocyte membrane permeability to K^+ (36, 37), but it is not yet clear whether this results in a net efflux (36, 37) or influx (38) of K^+ into the cell after mitogenic stimulation.

From these considerations, one attractive hypothesis is that the mitogenic trigger for lymphocytes involves changes in membrane potential and ion conductance across the membrane. Cone (39) has hypothesized that for somatic cells intracellular cation levels, associated with the generation of electrical membrane potential, may be involved in the control of mitogenesis and cell proliferation. In most instances for the lymphocyte, these ion events may be initiated by interaction and crosslinking of membrane receptors by mitogen, or antigen, and resemble action potentials generated by irritable tissue. In these latter tissues, ion currents are composed of calcium (and perhaps Na^+) influx and K^+ efflux from the cell. The possibility of Na^+ carrying the inward currents into the cell may explain why a reduction (equimolar for K^+) in the external sodium concentration in our experiments did not consistently override the

inhibitory effects of valinomycin on lymphocyte proliferation. Provided that the lymphocyte membrane is relatively impermeable to Na (i.e., low sodium permeability [P_{Na}]), this ion would not be expected to influence the resting membrane potential, but could contribute significantly to the depolarization currents.

Part or all of these inward currents could also be carried by calcium ions into the cytosol from either the external environment or from specialized compartments within the cell or cell membrane. It is noteworthy that calcium appears to be a necessary intracellular mediator or messenger in the secretory process of a number of cell systems, including the mitogenesis of lymphocytes (40, 41). This possibility is further supported for lymphocytes by the fact that the calcium ionophore, A23187, stimulates their blastogenesis and proliferation (4).

Taken together, these various observations and the present findings suggest that lymphocyte proliferation may be linked to the membrane potential and to ion movements across the membrane. Furthermore, ionophores, such as valinomycin and A23187, may serve as probes in examining the role of ions in lymphocyte activation and proliferation *in vitro*. Efforts along these lines seem worth pursuing, as there is increasing evidence that ionophores appear to be present in a number of normal tissues (42, 43), and naturally occurring ionophores could play a physiological role in the control of lymphocyte proliferation *in vivo*.

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