A POTASSIUM IONOPHORE (NIGERICIN) INHIBITS STIMULATION OF HUMAN LYMPHOCYTES BY MITOGENS*

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It has been proposed that an early and important event in mitogen-induced lymphocyte proliferation is a change in the intracellular concentration and/or transport of potassium ions (1). This hypothesis is supported by at least three lines of evidence: (*a*) immediately after lymphocyte stimulation by lectins, membrane Na-K ATPase activity is increased, causing an increase in the rate of potassium influx (2); (*b*) there is a concurrent increase in lymphocyte membrane permeability which leads, under certain conditions, to a loss of K^+ into the external medium (3–5); and (*c*) the addition of the cardiac glycoside, ouabain, a specific competitive inhibitor of the Na-K-ATPase pump, inhibits blastogenesis of T and B cells (6). Taken together, these various studies support the view that cation fluxes, and particularly K^+, may be of primary importance during the early events of lymphocyte stimulation.

In attempting to test this hypothesis, we have previously reported (7) that valinomycin, a specific potassium ionophore, inhibits phytohemagglutinin (PHA)[1]-stimulated blastogenesis and proliferation in human lymphocytes. The inhibition was not the result of cell toxicity, nor did it appear to be the result of its potential action as an uncoupler of oxidative phosphorylation (7), although the minimal concentrations of valinomycin which inhibit proliferation are close to those concentrations which show a slight effect on mitochondrial respiration (unpublished observations).

To inquire further into the role of K^+ fluxes across the cell membrane in lymphocyte mitogenesis, we have used another potassium ionophore, nigericin, which under appropriate conditions alters potassium distributions across biological membranes while negligibly affecting mitochondrial respiration (8, 9). In this study, the effects of nigericin on mitogen-stimulated proliferation and on oxidative phosphorylation were examined in human lymphocytes.

Materials and Methods

Lymphocyte Preparation. Heparinized blood (100–200 ml) was drawn from healthy adults and diluted in 1 vol of Hanks' balanced salt solution (HBSS) without calcium and magnesium.

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[1] *Abbreviations used in this paper:* Con A, concanavalin A; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; HBSS, Hanks' balanced salt solution; [^{3}H]dTR, tritiated thymidine; MEM, Eagle's minimal essential medium; MLC, mixed lymphocyte cultures; PHA, phytohemagglutinin; PWM, pokeweed mitogen.

Carbonyl iron (1–5 mg/ml blood) was added, and the mixture was shaken in a water bath for 45 min. In a modification of the method of Böyum (10), lymphocytes were isolated as previously described (11). The final cell suspension contained 0.5–2% polymorphonuclear leukocytes, 2–8% monocytes, 5–25% erythrocytes, and usually fewer than one platelet per lymphocyte. The purity of the lymphocyte preparation was determined by Wright-Giemsa staining (Harleco, Gibbstown, N. J.) and an esterase stain for monocytes (12) of cells dropped directly onto glass slides.

Lymphocyte Cultures. For studies of lymphocyte proliferation, cells were prepared as indicated above, except without carbonyl iron incubation. The resulting lymphocyte preparation contained 10–30% monocytes as determined by the esterase stain. Cultures were prepared by adding 5×10^5 cells to a 1.0-ml solution containing Eagle's minimal essential medium (MEM) plus pooled human AB serum (10% vol/vol) and concentrations of mitogens previously determined as optimal: 50 μg/ml of PHA-M (Difco Laboratories, Detroit, Mich.), 50 μg/ml of concanavalin A (Con A; Calbiochem, San Diego, Calif.), 20 μg/ml of pokeweed mitogen (PWM; Gibco Diagnostics, The Mogul Corp., Chagrin Falls, Ohio), or 2 μM of the calcium ionophore, A23187. Unless specified, all cultures except those stimulated with PWM were incubated at 37°C in 5% CO_2 in air for 72 h. The latter were terminated at 5 days and 0.25 μCi of tritiated thymidine ([^{3}H]dTR) (sp act 6.7 Ci/mmole; New England Nuclear, Boston, Mass.) was added to each culture 16 h before termination of the culture. Cultures were then harvested, and scintillation counting was performed by standard methods (11).

Nigericin and valinomycin (Sigma Chemical Co., St. Louis, Mo.) were added to lymphocyte cultures immediately before mitogens, and they were present throughout the culture period (except where otherwise specified). Both were 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 nigericin were tested by the trypan blue exclusion test and by cell counting (Coulter Electronics Inc., Hialeah, Fla.) using a modification of the cetrimide technique (13).

In certain experiments, lymphocytes were cultured in MEM supplemented with KCl. In all experiments, the external medium contained 1.3 mM of Ca and 1.0 mM of Mg, 0.3 mM HPO_4^{-2}, 1.3 mM $H_2PO_4^-$, 1.4 mM bicarbonate, 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.

Mitotic Counts. In experiments comparing mitotic counts to uptake of [^{3}H]dTR, parallel cultures were exposed to colchicine (0.1 μg/ml) for 6–8 h before termination and fixed with methanol/acetic acid (3:1) after hypotonic swelling in 0.075 M of sodium citrate and 10% serum. Mitotic indexes were determined from triplicate slides stained with Giemsa; 1,000 cells/slide were counted. The total mitoses per culture were calculated from the mitotic index and cell survival data.

Mixed Lymphocyte Cultures (MLC). Lymphocytes were obtained from unrelated donors and prepared (as were mitogen cultures) on a Ficoll-Hypaque gradient as described above. Cells (2.5×10^5) from each donor were incubated in 1.0 ml MEM with 10% AB serum, at 37°C in a humidified atmosphere containing 5% CO_2 in air for 5 days. The cultures were harvested, and scintillation counting was performed as described above. In certain experiments, nigericin was added at the specified concentration at the outset of the cultures.

Oxygen Consumption. Oxygen consumption of purified lymphocytes was measured polarographically with a Clark electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio). Purified lymphocytes (>90%) were suspended at 30×10^6 cells/ml in HBSS in a 1.0-ml glass chamber (Gibson Electric, Inc., Delmont, Pa.) and O_2 determinations were performed at 22°C with constant stirring of the suspension. In certain experiments, O_2 determinations were made after cells were exposed to nigericin, valinomycin, or carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP; Sigma Chemical Co.).

Lymphocyte ATP. ATP was measured using the bioluminescence method (luciferase) as described by Kimmich et al. (14) with the exception that firefly-tail extract (Sigma Chemical Co.) was used. Cells were suspended at 5×10^6/ml in HBSS buffered with 20 mM *N*-2-hydroxyethylpiperazine-*N'*-ethane sulfonic acid, and ATP was measured after incubation for 20 min at 22°C or 24 h at 37°C, with or without nigericin.

Results

The Inhibitory Effect of Nigericin on Thymidine Uptake. Fig. 1 A shows

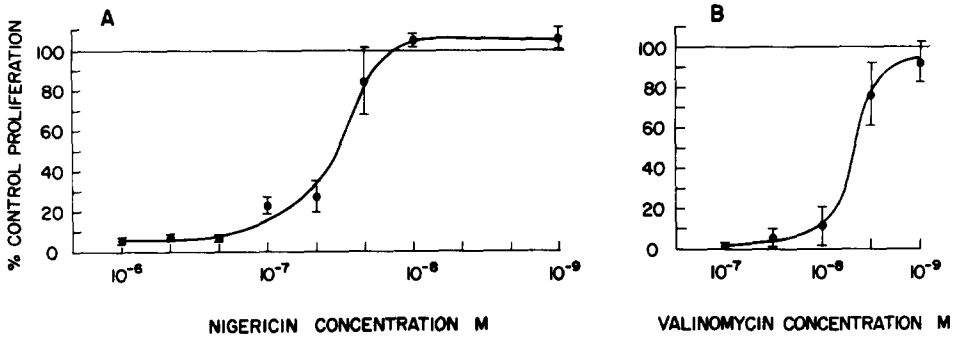

FIG. 1. (A) The effect of various concentrations of nigericin on the inhibition of lymphocyte mitogenesis measured by the incorporation of [^{3}H]dTR at 72 h. [^{3}H]dTR incorporation for control cultures (100%) equaled 67,494 ± 10,525 for six experiments (mean ± standard deviation). Each point equals the mean ± standard deviation. Curve was fitted by eye. (B) The effect of various concentrations of valinomycin on the inhibition of lymphocyte mitogenesis measured by the incorporation of [^{3}H]dTR at 72 h. [^{3}H]dTR incorporation for control cultures (100%) equaled 65,500 ± 13,700 for 10 experiments (mean ± standard deviation). Each point equals the mean ± standard deviation. Curve was fitted by eye. [Data were previously reported (7)].

the results of six experiments testing various concentrations of nigericin (10^{-6}–10^{-9} M) on PHA-stimulated lymphocyte proliferation as measured at 72 h. At nigericin concentrations of 10^{-6} and 5×10^{-7} M, there was complete inhibition of the proliferative responses as compared to controls. At concentrations of 2×10^{-7}–5×10^{-8} M, there was 80–90% inhibition. Between 5×10^{-8} and 10^{-8} M of nigericin, there was an abrupt decrease in the effect and no inhibition was observed at 10^{-9} M or less.

In comparing the inhibitory effects of nigericin to valinomycin (Fig. 1 B), both agents were inhibitory at concentrations of 10^{-6}–5×10^{-8} M and both had a sigmoid dose-response curve. An abrupt decrease in the inhibition of PHA responses occurred at 2.5×10^{-8} and 5×10^{-9} M for nigericin and valinomycin, respectively.

Inhibitory Effect of Nigericin on Lymphocyte Mitoses. To determine if the increase in [^{3}H]dTR uptake correlated with an actual decrease in cell proliferation, additional studies were performed in which the number of mitoses were determined in mitogen-stimulated cultures after 72 h in the presence or absence of nigericin. As shown in Table I, the decrease in mitotic index paralleled the decrease in [^{3}H]dTR uptake.

The Effect of Nigericin on Lymphocyte Survival. Lymphocytes were cultured over a 72-h period in the presence or absence of various concentrations of nigericin. Lymphocyte survival was determined at 24, 48, and 72 h. There was no significant decrease in cell survival in the presence of 10^{-6}–10^{-7} M nigericin over a 72-h period as compared to controls.

Reversibility of Nigericin Effects. Lymphocytes were incubated with nigericin plus PHA for either 4 or 24 h, washed twice in MEM, and then tested for their proliferative response to PHA 72 h later. Control PHA cultures, lacking nigericin, were similarly washed after 4 and 24 h incubation. Lymphocytes treated with inhibitory concentrations of nigericin for periods of 4 or 24 h were capable of almost complete recovery of PHA responsiveness (100 and 75%, re-

TABLE I
Effect of Nigericin on Lymphocyte Mitosis in PHA-Stimulated Cultures

Culture	Mitoses per 1,000*	Cell survival ($\times 10^5$)	Total mitoses*	[^{3}H]dTR Uptake*
				cpm
Control (no PHA)	1 ± 0.3	3.63	242 ± 121	183 ± 26
PHA	40 ± 2	5.50	21,820 ± 112	72,509 ± 2,215
PHA + 5×10^{-7} M nigericin	8 ± 5	3.29	2,413 ± 915	5,046 ± 403
PHA + 2.5×10^{-7} M nigericin	3 ± 1	2.96	888 ± 452	6,903 ± 960

* Values are expressed as mean values ± standard error of the mean of triplicate cultures.

spectively) as compared to control cultures. The reversibility (in additional experiments), after washing both drug and mitogen from the cultures after a 24-h incubation without subsequent replacement of PHA also suggests that nigericin does not act by interfering with the binding of PHA to lymphocytes.

Metabolic Effects of Nigericin. Under certain circumstances, nigericin may uncouple oxidative phosphorylation, leading to a decrease in cellular ATP (15). In our system, oxygen uptake and the total cellular concentration of ATP were measured after incubating purified lymphocytes (>90%) in the presence of various concentrations of nigericin under conditions comparable to those of mitogen-stimulated cultures. Over a 10^{-5}–10^{-7} M range of concentrations of nigericin, there was a negligible change in cellular respiration. In contrast, lymphocyte oxygen consumption increased in the presence of 10^{-5}–10^{-6} M FCCP and 10^{-6} M valinomycin—known uncouplers of oxidative phosphorylation.

Taking advantage of the bioluminescence assay (luciferase), the total concentration of lymphocyte ATP was measured after incubation for 20 min or 24 h at 37°C in the presence of various concentrations of nigericin (10^{-6}–10^{-8} M). As shown in Tables II and III, there was no significant decrease in the ATP concentration of lymphocytes exposed for 20 min or 24 h to nigericin. Interestingly, however, there was a significant increase in the cellular ATP concentration after 24 h incubation with 10^{-8} M nigericin.

Time of Inhibition. Nigericin was added to mitogen-stimulated cultures at various times after initiation of the cultures; proliferation was measured at 72 h. Fig. 2 depicts the results of two experiments on separate donors and is representative of five experiments. Inhibition of lymphocyte responses by nigericin (10^{-7} M) occurred when nigericin was added to the cultures between 0 and 21 h. With addition at 24 h, the proliferative response was minimally reduced (80 and 100% of control); when added after 24 h, there was no measurable decrease in the proliferative response. In experiment 2, mitotic indexes were also determined, and these paralleled the changes observed in the uptake of [^{3}H]dTR.

The Effect of External Potassium on the Inhibitory Effects of Nigericin. Although increasing potassium concentration in the external medium up to 50 mM did not reverse the inhibitory effects of nigericin (Fig. 3 A), we confirmed our previous observations that increasing the K^+ of the external medium reversed the inhibitory effect of valinomycin (Fig. 3 B). The osmolality

TABLE II
*Lymphocyte ATP Content (2 h)**

Experiment	Control	Nigericin (M)				
		10^{-6}	5×10^{-7}	10^{-7}	5×10^{-8}	10^{-8}
1	122	114	114	118	127	–
2	47	52	42	47	43	52
3	41	45		57		61
4	95			111		130
Mean ± SE	76 ± 19	70 ± 22	78	83 ± 18	85	81 ± 25

Culture conditions: 20 min at 22°C with 10% AB serum.
* Each value equals pM ATP/5 × 10^6 cells and is the mean of triplicate samples.

TABLE III
*Lymphocyte ATP Content (24 h)**

Experiment	Control	Nigericin (M)		
		10^{-6}	10^{-7}	10^{-8}
1	29	31	33	57
2	46	32	40	81
3	41	45	57	62
Mean ± SEM	38 ± 5	36 ± 5	43 ± 7	67 ± 7‡

* ATP content measured as pM ATP/5 × 10^6 cells after 24 h incubation at 37°C in 10% AB serum (with and without nigericin).
‡ Difference between this value and control is significant, $P < 0.002$ (paired Student's t test). Values are the mean of triplicate samples.

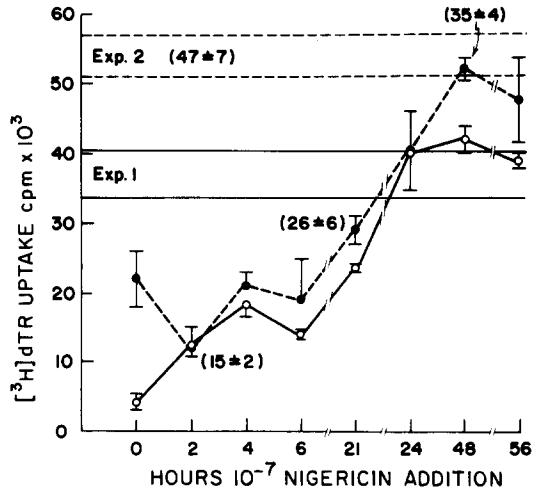

FIG. 2. The effect of adding nigericin (10^{-7} M) at various times after the initiation of PHA cultures. PHA was added at 0 time and the proliferative response was measured 72 h later. Each point equals the mean ± standard error of the mean for triplicate cultures. Areas included in the closed and dotted lines represent mean ± standard error of the mean of control cultures lacking nigericin; (○), experiment 1; (●), experiment 2. The values in parentheses equal the number of mitoses/1,000 cells (mean ± standard error of the mean for triplicate cultures) determined in parallel cultures.

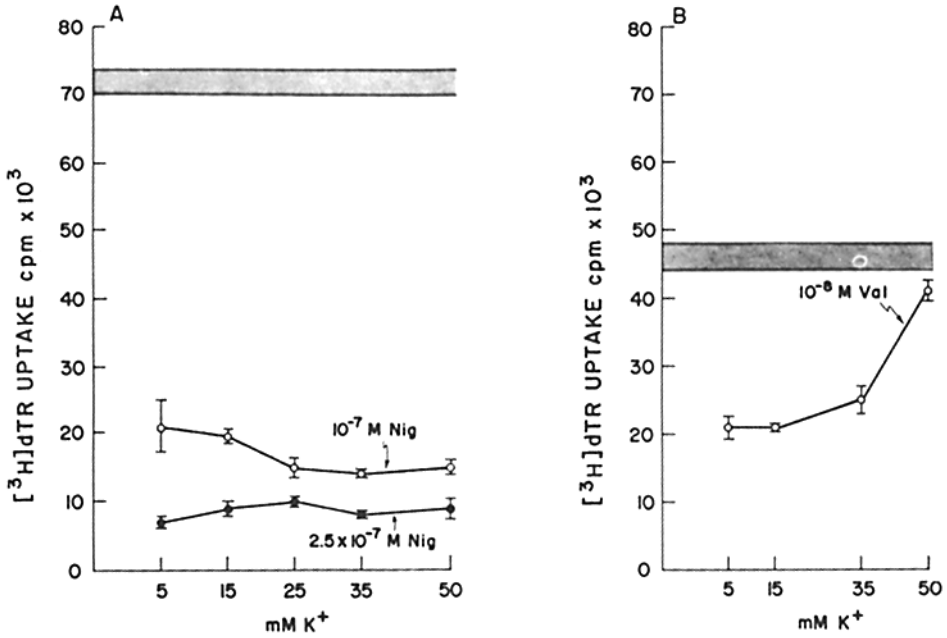

FIG. 3. (A) The effect of increasing the potassium concentration of the external medium on [^{3}H]dTR incorporation at 72 h for lymphocytes cultured in the presence of nigericin (Nig). (○), 10^{-7} M nigericin; (●), 2.5×10^{-7} M nigericin. Each point equals the mean ± standard error of the mean of triplicate cultures. Shaded area equals control cultures (no nigericin). (B) The effect of increasing the potassium concentration of the external medium on [^{3}H]dTR incorporation at 72 h for lymphocytes cultured in the presence of 10^{-8} M valinomycin (Val). Each point equals the mean ± standard error of the mean of triplicate cultures. Shaded area equals control cultures (lacking valinomycin).

of MEM supplemented with KCl equaled 290 and 380 mosmol for 5 and 50 mM K^+, respectively. Neither cell viability nor the capacity of lymphocytes to respond to PHA was impaired when cultured in MEM supplemented with these concentrations of KCl (data not shown).

The Effect of Nigericin on Other Mitogens and on the MLC. As indicated in Fig. 4 A, 4 B, and 5 A, nigericin inhibited the proliferative responses to Con A, PWM, and A23187, but at concentrations that were different from those which inhibited responses to PHA. For example, nigericin inhibited A23187 stimulation of lymphocytes at 10^{-8} M and also in subsequent experiments in which nigericin was decreased to 10^{-9} M (data not shown).

To test the effects of nigericin on T-cell proliferation in a system which involves antigenic stimulation, two-way MLC were studied. As occurred with mitogen responses, concentrations of nigericin from 5×10^{-7} to 10^{-8} M inhibited the MLC response (Fig. 5 B).

Discussion

These studies demonstrate that nigericin, an ionophore that exchanges K^+ for H^+, reversibly inhibits stimulation of human lymphocytes by mitogens. This effect was dose-dependent, with significant inhibition at concentrations of

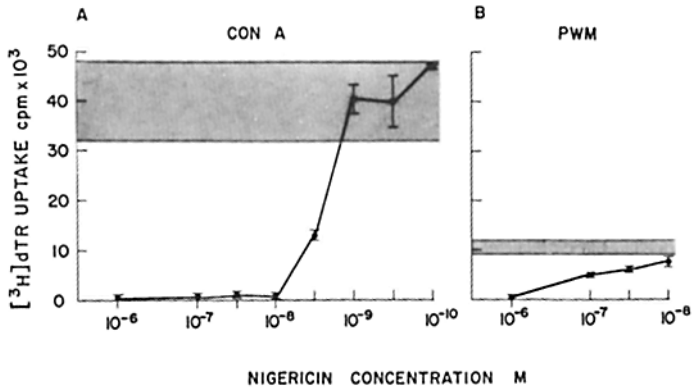

FIG. 4. (A) The effect of various concentrations of nigericin on lymphocyte stimulation by Con A. Each point equals the mean ± standard error of the mean for triplicate cultures at 72 h. Shaded area equals control cultures lacking nigericin. (B) The effect of various concentrations of nigericin on the proliferative response of lymphocytes to PWM measured at 5 days. Each point equals the mean ± standard error of the mean for triplicate cultures. Shaded area equals values for control cultures lacking nigericin.

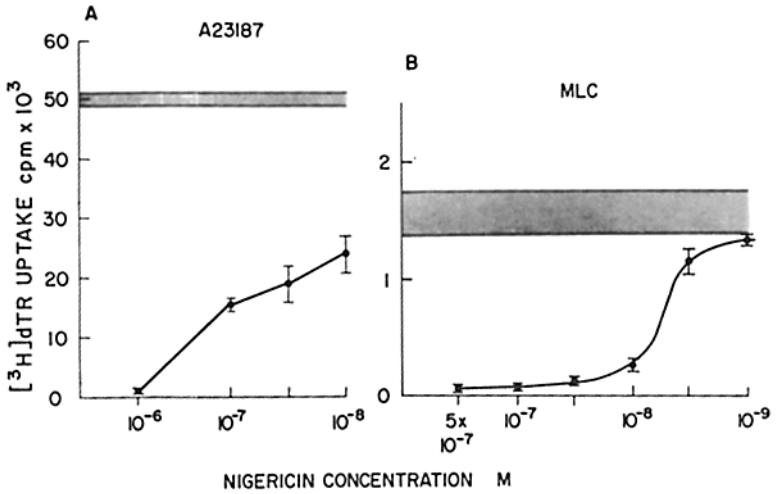

FIG. 5. (A) The effect of various concentrations of nigericin on the proliferative response of lymphocytes to the calcium ionophore A23187, measured at 72 h. Each point equals the mean ± standard error of the mean of triplicate cultures. Shaded areas equal control cultures lacking nigericin. (B) The effect of various concentrations of nigericin on a two-way MLC; the proliferative response was measured at 5 days. Each point equals the mean ± standard error of the mean for triplicate cultures. Shaded areas are MLC cultures lacking nigericin. Line was fitted by eye.

10^{-6}–10^{-8} M. The shape of the dose response curve, as well as concentrations at which nigericin was inhibitory, were comparable to results obtained previously with valinomycin, another potassium ionophore (7).

Nigericin appears to exert its effect early in the sequence of events leading to lymphocyte proliferation. Addition of nigericin at 24 h or later after the initiation of PHA cultures did not result in significant inhibition. The inhibitory

effects of nigericin could not be ascribed to cell killing, nor does nigericin appear to act by inhibiting mitochondrial respiration. Oxygen uptake of lymphocytes treated with nigericin differed only slightly from that in untreated control cultures. Furthermore, the ATP content of lymphocytes cultured in the presence of nigericin for 20 min, or 24 h was not decreased compared to controls.

If the inhibitory effects of nigericin, and also valinomycin (7), are not the result of uncoupling, then it is possible that they act by altering the distribution and/or permeability of cations across the cell membrane. Nigericin and valinomycin can both act as potassium ionophores, but important differences exist in the proposed mechanisms by which they translocate potassium across biological membranes (8, 9). Valinomycin forms a lipid-soluble, positively charged complex with potassium. The complex diffuses rapidly across the lipid phase of both artificial and natural membranes allowing potassium to equilibrate with an aqueous phase. Thus, valinomycin catalyzes the passive movement of potassium across the membrane in the direction of the electrochemical potential and can mediate an electrogenic movement of ions.

In contrast, nigericin, a monocarboxylic acid, catalyzes an electroneutral exchange of alkali metals for hydrogen ions. Although nigericin prefers potassium, the ion selectivity for K^+ (8) is not as great as that with valinomycin. Since nigericin is considered to carry out an electroneutral exchange, it does not usually alter membrane potential or uncouple oxidative phosphorylation. Based on these considerations, several possible mechanisms may account for the inhibitory effect of nigericin on lymphocyte proliferation.

Decreases in Intracellular Potassium. Nigericin might act by causing a critical drop in intracellular potassium as K^+ moves down a concentration gradient and out of the cell. Evidence suggests that a homeostatic mechanism may exist so that increased activity of the Na-K-ATPase pump after mitogen stimulation maintains the intracellular potassium despite an increase in cell membrane permeability and efflux of potassium ions (4). Macromolecular synthesis in mammalian cells is apparently dependent upon maintenance of the intracellular potassium concentration (16).

In this study, however, nigericin exerts its effect only during the initial 24 h of lymphocyte cultures. If a critical drop in intracellular potassium were the only cause for the inhibitory effect, then nigericin should also act at a time of maximal macromolecular synthesis (e.g., 48–72 h after stimulation). Furthermore, as with valinomycin (7), the inhibitory effects of nigericin should be reversed by increasing the K^+ concentration of the external medium.

Changes in Membrane Potential. The mitogenic trigger for lymphocytes may involve changes in membrane potential related to changes in ion permeability. For somatic cells, Cone (17) has provided evidence that intracellular cation levels, associated with the generation of membrane potential, may be involved in the control of mitogenesis and cell proliferation. We have proposed a similar mechanism for lymphocyte proliferation (7). Assuming that nigericin effects an electroneutral exchange of potassium for hydrogen ions in most systems, it would not be expected to alter the membrane potential. It is not known, however, whether or not nigericin can serve as a potassium conductor (15) or alter the membrane permeabilities of other cations which may be

important in determining lymphocyte membrane potential. Thus, its ability to alter membrane potential remains a possibility.

Changes in pH Gradient. Recent studies by Johnson et al. (18) have demonstrated that decreases in intracellular H^+ concentration are required for the activation and development of sea urchin eggs after fertilization. These findings suggest that pH gradients across the cell membrane may be important in the regulation of cell division. An electroneutral exchange of potassium for hydrogen ions across the lymphocyte membrane mediated by nigericin, could alter the pH gradient across the membrane, resulting from an increase in intracellular H^+ concentration, and this could perhaps influence lymphocyte mitogenesis.

Interaction of Nigericin with the Cell Membrane. A final possibility is that inhibition may result from nigericin incorporation into the lipid phase of the lymphocyte membrane, rather than its action on cation flux or membrane permeability. Intercalation of nigericin within the membrane could, in some way, alter the arrangement of membrane determinants which would block a mitogenic stimulus or induce an inhibitory signal. Such a mechanism has recently been advanced to explain the mitogenic properties of certain polymeric ionophores for mouse lymphocytes (19).

As a related possibility, it might be argued that the effects of nigericin are the result of inhibition in the binding of PHA to the lymphocyte membrane. This is unlikely since: (*a*) other lectins (Con A, PWM) were inhibited to an equal extent by nigericin; (*b*) nigericin inhibited the MLC; (*c*) nigericin inhibited the calcium ionophore A23187, which is presumed to bypass interaction with membrane glycoproteins in triggering lymphocyte proliferation (20, 21); and (*d*) mitogen responses were preserved after incubation (0–24 h) and removal of both PHA and nigericin from the cell cultures.

Although it is tempting to ascribe the inhibitory effects of both nigericin and valinomycin to a common mechanism involving an alteration of K^+ flux across the cell membrane, it would seem premature to favor one hypothesis strongly. Each of the possibilities discussed above is currently being investigated.

At least two implications emerge from these data and theoretical considerations. First, potassium ionophores, such as nigericin and valinomycin, may serve as probes in determining the role of potassium, cation fluxes, and perhaps membrane potential in the events leading to lymphocyte proliferation. Second, it is possible that potassium (or other cation) ionophores may be prototypes for, or mimic the action of, other inhibitory substances (e.g., chalones) (22) which regulate the proliferation of lymphocytes and other cells in vivo. The recent identification of naturally occurring ionophores in tissues such as heart and kidney (23, 24) raises the possibility that some of the substances, such as macrophage products, which may influence lymphocyte proliferation in vivo (25, 26), are related to these experimental models involving ionophores.

Summary

Nigericin, an ionophore that exchanges K^+ for H^+ across most biologic membranes, reversibly inhibited the proliferative response of human lymphocytes to phytohemagglutinin (PHA). Inhibition occurred at nigericin concentra-

tions of 10^{-8} M or greater, and only during the early event of mitogenesis. There was no effect if nigericin was added 24 h or later after the initiation of PHA-stimulated cultures. The effect was not the result of toxicity or impaired mitochondrial respiration. At similar concentrations, nigericin also inhibited lymphocyte responses in mixed lymphocyte cultures and to other mitogens including concanavalin A, pokeweed mitogen, and the calcium ionophore, A23187. The findings support the view that one or more transmembranous events, mediated by changes in cation flux and/or membrane potential, are critical in the initial stages of lymphocyte mitogenesis.

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