It would be of interest to know if the plasma amine oxidase is inhibited in actual cases of lead poisoning. While it is difficult to draw conclusions regarding the effects of lead *in vivo* based on these *in vitro* results, a rough calculation can be made. In patients with clear evidence of lead toxicity, blood levels of lead range from 100 to 1000 μg Pb^{2+}/100 ml whole blood [25]. More than 90% of the lead is believed to sequester in erythrocytes [26]. Thus, 0.5 to 5 μM lead would remain in serum. Not all of this lead would be available to inhibit the amine oxidase because other serum proteins such as albumin can bind Pb^{2+} [20]. Our data indicate that the amine oxidase activity inhibited 10% in the presence of 25 μM Pb^{2+}. While it seems unlikely that this enzyme would be inhibited by lead levels sufficient to cause the clinical disorder, the definitive answer can only come from studies correlating enzyme activity with blood lead levels.

In summary, Pb^{2+} acted as a noncompetitive, reversible inhibitor of bovine plasma amine oxidase *in vitro*. The inhibition constant (K_i) for $Pb(NO_3)_2$ was 46 μM.

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Effect of valinomycin on human peripheral blood lymphocytes

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Valinomycin, a cyclic dodecadepsipeptide that enhances ion permeability of artificial membranes selectively for K^+, has been reported to inhibit phytohemagglutinin (PHA)-induced lymphocyte transformation in human peripheral blood lymphocytes [1, 2] as judged by depressed [^{3}H]thymidine ([^{3}H])TdR) incorporation into DNA. The inhibitory effect of valinomycin on mitogenic transformation was overcome by increasing the external K^+, suggesting that interaction of PHA with specific receptors on the lymphocyte cell membrane may involve mechanisms affecting cation fluxes and membrane potential [2]. The mechanism of inhibition of mitogenesis by valinomycin remains unsettled.

The present investigation was designed to ascertain whether non-lectin mitogens, which do not require the presence of specific binding sites for their action, would also be unable to induce blastogenesis in the presence of valinomycin. The inhibitory action of valinomycin may occur at a step(s) subsequent to initial stimulation and be totally non-specific as to the agent or technique used to induce transformation. The effect of increasing the external K^+ concentration on inhibition of mitogenesis by valinomycin was also examined, along with the effect of valinomycin on [^{3}H]TdR and [^{3}H]uridine incorporation by maximally transformed cells, in order to distinguish between the degree of actual inhibition of lymphocyte

transformation and any inhibitory effects of valinomycin on the uptake of the labeled nucleosides or subsequent reactions leading to DNA and RNA syntheses.

Materials and methods

Valinomycin (Sigma Chemical Co., St. Louis, MO) was dissolved in absolute ethanol; the concentration of ethanol to which cells were exposed did not exceed 0.2%. Cells cultured in the absence of valinomycin were also exposed to 0.2% ethanol. Viability of cells after exposure to all concentrations of valinomycin was monitored by the ability to exclude trypan blue dye. Viability was always greater than 95%. The K^+ concentration of the culture medium was determined by flame photometry. Peripheral blood was obtained from healthy donors and collected in preservative-free heparin (20 units/ml blood). Lymphocytes were isolated by centrifugation over a Ficoll–Hypaque gradient at 400 *g* for 30 min [3]. Periodate-, PHA- and A23187-induced DNA synthesis and blast formation were performed according to previously described methods [4]. RNA synthesis assay was performed according to the procedure of Pienkowski *et al.* [5] with minor modifications. Cells were suspended for culture in RPMI-1640 supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), amphotericin B (0.23 μg/ml) and 10% fetal calf serum. Cultures (1×10^6 cells) were prepared in triplicate and incubated with 5 μg PHA and 2.5 μCi [^{3}H]uridine (30 Ci/mmole) in a final volume of 1.0 ml for periods of time varying between 4 and 48 hr.

Results and discussion

To determine whether valinomycin affects the transformation of small lymphocytes to large blastoid cells, it was added at various concentrations to cell cultures 4 hr prior to addition of PHA or A23187 and immediately following washing and suspension of periodate-oxidized cells. The effect of valinomycin on blast transformation was observed at the maximal stimulation response times of PHA, A23187 (72 hr) and periodate (48 hr). In the absence of valinomycin, PHA stimulation resulted in the highest number of blast cells produced (88%) compared to periodate (65%) and A23187 (34%). The percentages of blast cell formation in the presence of valinomycin concentrations of 2×10^{-9} M, 2×10^{-8} M, 2×10^{-7} M, and 2×10^{-6} M were 41, 36, 19 and 11% for periodate-treated cells; 78, 37, 20 and 3% for PHA-treated cells; and 28, ND, 25, and 4% for A23187-treated cells respectively.

The results of several experiments that indicate the effects of various concentrations of valinomycin on periodate-, A23187- and PHA-stimulated lymphocyte proliferation are shown in Table 1. Valinomycin was added to lymphocyte cultures after maximal stimulation with periodate, A23187 or PHA to measure the effect of the valinomycin on transformed cells' uptake of [^{3}H]TdR and prior to cell stimulation when investigating its effect on the transformation of the peripheral blood lymphocytes. When valinomycin was added to cultures 72 hr after PHA stimulation, suppression of [^{3}H]TdR incorporation was observed. At valinomycin concentrations of 10^{-6} M and 10^{-7} M there were decreases of 51 and 58%, respectively, in [^{3}H]TdR incorporation compared to cells cultured in the absence of valinomycin. A 10^{-8} M concentration of valinomycin resulted in a slight inhibition. Addition of valinomycin prior to exposure of cells to PHA inhibited the mitogen-induced incorporation of [^{3}H]TdR in a concentration-dependent manner. Results similar to that with PHA were obtained when valinomycin was added prior to and following stimulation with A23187 and periodate.

As seen in Table 2, valinomycin severely inhibited PHA-induced RNA synthesis in a concentration-dependent manner. PHA-treated lymphocytes exposed to 10^{-8} M or greater concentrations of valinomycin incorporated levels of [^{3}H]uridine similar to that obtained in unstimulated (minus mitogen) cells cultured in the presence of the same concentration of valinomycin. The level of [^{3}H]uridine incorporation by cells not exposed to mitogen was also adversely affected by the presence of increasing amounts of valinomycin. A 41% decrease in [^{3}H]uridine incorporation by cells stimulated in the presence of 10^{-8} M valinomycin (3299 cpm) compared to cultures stimulated in the absence of the drug (5628 cpm) was measured as early as 4 hr after stimulation (not shown). The effect of

Table 1. Effect of valinomycin on periodate-, A23187-, and PHA-induced lymphocyte transformation and on the uptake of [^{3}H]TdR by transformed lymphocytes

Additions	[^{3}H]TdR incorporated (mean cpm ± S.D.) Valinomycin added prior to stimulation	Valinomycin added to fully stimulated cultures
None	1,530 ± 60	1,480 ± 70
Periodate alone	11,870 ± 930 (100)*	11,460 + 100 (100)
Periodate plus		
2×10^{-9} M Valinomycin	9,950 ± 1,240 (84)	10,060 ± 520 (88)
2×10^{-8} M Valinomycin	410 ± 30 (3)	7,130 ± 60 (62)
2×10^{-7} M Valinomycin	320 ± 60 (3)	7,840 ± 440 (68)
2×10^{-6} M Valinomycin	300 ± 40 (2)	7,300 ± 780 (64)
None	2,190 ± 210	2,440 ± 160
A23187 alone	8,600 ± 2,160 (100)	11,100 ± 1,140 (100)
A23187 plus		
2×10^{-9} M Valinomycin	8,760 ± 2,160 (102)	10,550 ± 230 (95)
2×10^{-8} M Valinomycin	160 ± 10 (2)	8,310 ± 1,220 (75)
2×10^{-7} M Valinomycin	200 ± 70 (2)	7,190 ± 2,040 (65)
2×10^{-6} M Valinomycin	190 ± 40 (2)	7,690 ± 2,200 (69)
None	7,190	6,470 ± 300
PHA alone	155,730 ± 46,210 (100)	142,670 ± 1,730 (100)
PHA plus		
1×10^{-8} M Valinomycin	106,820 ± 9,970 (68)	124,700 ± 13,260 (87)
1×10^{-7} M Valinomycin	28,470 ± 14,670 (18)	60,060 ± 9,930 (42)
1×10^{-6} M Valinomycin	10,190 ± 5,361 (6)	69,600 ± 9,900 (49)

* Values shown in parentheses correspond to the percentage of [^{3}H]TdR incorporation, taking as 100% that obtained in the presence of mitogen alone.

valinomycin was not due to a delayed response, but rather to decreased RNA synthesis, the kinetics of which indicate that the degree of inhibition increased with time of incubation after PHA addition (not shown). When valinomycin was added to lymphocyte cultures at a concentration of 5×10^{-8} M after maximal stimulation with PHA to measure the effect of valinomycin on transformed cells' incorporation of [^{3}H]uridine into RNA, there was a 59% decrease in [^{3}H]uridine incorporation compared to cultures where valinomycin was omitted. Decreases of 33 and 19% in [^{3}H]uridine incorporation were measured at 10^{-8} M and 5×10^{-9} M valinomycin respectively.

Lymphocytes were cultured with different concentrations of valinomycin (2×10^{-9} M to 2×10^{-6} M) in which the K^+ concentration of the external medium was 5.5×10^{-3} M and in the same medium supplemented with KCl to bring the concentration to 41×10^{-3} M. The results (Table 3) show that, in the absence of valinomycin, PHA stimulation of lymphocytes suspended in culture medium containing 41×10^{-3} M K^+ incorporated 3.8-fold more [^{3}H]TdR than identically treated cells stimulated and cultured in the same medium containing 5.5×10^{-3} M KCl. A 2-fold increase in total [^{3}H]TdR incorporation at 41×10^{-3} M K^+ compared to 5.5×10^{-3} M K^+ (in the absence of valinomycin) was obtained with periodate-oxidized cells (Table 3, Exp. 2). The higher K^+ concentration did not alleviate the inhibition of [^{3}H]TdR incorporation by valinomycin of PHA- or periodate-induced lymphocyte transformation. Raising the concentration of K^+ in RPMI-1640 to that found in the lymphocyte (120×10^{-3} M) would result in a K^+ gradient of zero and reversal of the valinomycin effect might occur. [^{3}H]Uridine and [^{3}H]TdR incorporation by resting and mitogen-stimulated cells, however, was completely blocked at this concentration of external K^+ both in the presence and absence of valinomycin.

Table 2. Effect of valinomycin on changes in RNA synthesis associated with PHA stimulation of lymphocytes

Additions	[^{3}H]Uridine incorporated (mean cpm ± S.D.)
None	18,770 ± 1,600 (100)*
1×10^{-9} M Valinomycin	17,620 ± 780 (94)
5×10^{-9} M Valinomycin	15,830 ± 3,550 (85)
1×10^{-8} M Valinomycin	13,280 ± 770 (71)
5×10^{-8} M Valinomycin	12,730 ± 700 (67)
1×10^{-7} M Valinomycin	12,800 ± 940 (68)
5×10^{-7} M Valinomycin	11,250 ± 960 (60)
PHA (5 μg/ml) alone	56,880 ± 10,200 (100)
PHA (5 μg/ml) plus	
1×10^{-9} M Valinomycin	53,090 ± 7,010 (93)
5×10^{-9} M Valinomycin	36,910 ± 12,210 (65)
1×10^{-8} M Valinomycin	21,830 ± 3,340 (38)
5×10^{-8} M Valinomycin	19,030 ± 4,600 (33)
1×10^{-7} M Valinomycin	17,890 ± 1,460 (31)
5×10^{-7} M Valinomycin	12,010 ± 2,160 (21)

* Values shown in parentheses correspond to the percentage of [^{3}H]uridine incorporation with the value obtained in the absence of valinomycin taken as 100%.

Daniele and Holian [2] reported that increasing the K^+ concentration of the culture medium to 50×10^3 M does not result in enhanced [^{3}H]TdR incorporation in cells stimulated with PHA in the absence of valinomycin when compared to cells cultured in standard MEM containing 5.5×10^{-3} M K^+. We have consistently observed enhanced incorporation of labeled nucleoside in cells cultured in media (MEM and RPMI-1640) supplemented with KCl.

Daniele and Holian [6] reported that 10^{-7} M valinomycin inhibits anti-immunoglobulin-induced cap formation in human peripheral blood lymphocytes and that this inhibition is blocked by increased external K^+. Montecucco *et al.* [7] have described inhibition of capping in mouse spleen cells by 10^{-7} M valinomycin. The inhibition was not blocked by increasing the K^+ concentration of the cell-suspending medium, suggesting that valinomycin may not be exerting its effect at the cell membrane.

The inhibitory effect of valinomycin on lymphocyte mitogenesis [1, 2] was determined by measuring [^{3}H]TdR incorporation into DNA. An effect of valinomycin on the [^{3}H]-TdR incorporation assay was not ruled out in these studies. In this report, the effect of valinomycin on [^{3}H]-uridine and [^{3}H]thymidine incorporation in already transformed cells and in resting lymphocytes was measured to assess the degree of actual inhibition of lymphocyte transformation from any inhibitory effects of valinomycin on incorporation of the labeled nucleosides. Significant decreases (25–58%) in [^{3}H]TdR incorporation by cells pre-stimulated with optimal mitogenic concentrations of PHA,

Table 3. Effect of potassium ion concentration of culture medium on PHA- and periodate-stimulated lymphocytes cultured in the absence and presence of various concentrations of valinomycin

Additions	[^{3}H]TdR incorporated (mean cpm ± S.D.) RPMI-1640 medium containing: 5.5 mM K^+	41 mM K^+
None	26,080 ± 2,910	39,660 ± 660
PHA alone	86,530 ± 3,220 (100)*	325,452 ± 45,410 (100)
PHA plus		
2×10^{-9} M Valinomycin	31,420 ± 1,810 (36)	176,410 ± 52,790 (54)
2×10^{-8} M Valinomycin	29,930 ± 6,520 (35)	66,770 ± 9,460 (20)
2×10^{-7} M Valinomycin	11,430 ± 2,670 (13)	36,310 ± 4,890 (11)
2×10^{-6} M Valinomycin	16,410 ± 2,990 (19)	44,440 ± 4,680 (14)
None	2,850 ± 140	3,320 ± 200
Periodate alone	7,110 ± 1,380 (100)	14,700 ± 600 (100)
Periodate plus		
2×10^{-9} M Valinomycin	5,080 ± 450 (71)	10,080 ± 1,200 (68)
2×10^{-8} M Valinomycin	790 ± 820 (11)	4,570 ± 1,860 (31)
2×10^{-7} M Valinomycin	260 ± 80 (4)	950 ± 130 (6)
2×10^{-6} M Valinomycin	130 ± 20 (2)	540 ± 70 (4)

* Values shown in parenthesws correspond to the percentage of [^{3}H]TdR incorporation with the value obtained in the absence of valinomycin taken as 100%.

A23187 or periodate were observed at valinomycin concentrations of 10^{-8} M and 10^{-7} M. The same concentrations of valinomycin when added to cell cultures prior to stimulation resulted in almost complete inhibition of [^{3}H]TdR uptake, demonstrating the inhibitory effect of valinomycin on mitogen-induced lymphocyte transformation. The inhibitory effect of valinomycin on labeled nucleoside incorporation by the lymphoblasts and resting lymphocytes may result from (a) impairment of nucleoside uptake or transport in the cell or (b) interference of subsequent reactions leading to DNA or RNA syntheses.

The results presented in this report suggest that caution should be used when interpreting some of the results in earlier studies [1, 2] using valinomycin to examine the role of K^+ in PHA-induced lymphocyte transformation. This is based on (a) our inability to overcome the inhibitory effect by valinomycin of lymphocyte transformation by raising the external K^+ concentration and (b) interference with RNA synthesis in resting lymphocytes and DNA and RNA syntheses in maximally stimulated lymphocytes as judged by depressed incorporation of [^{3}H]TdR and [^{3}H]uridine. The actual degree of interference of lymphocyte transformation by valinomycin is difficult to assess because of these previously unknown "side effects" of valinomycin on lymphocyte metabolism.

Valinomycin has also been shown to affect mitochondrial function in intact, resting peripheral blood lymphocytes. Exposure of cells to the ionophore results in decreased cellular ATP and increased oxygen consumption [8–10]. These studies were performed using valinomycin concentrations of 10^{-6} M and greater. The effect of valinomycin in the concentration range used in our study on these parameters of cellular metabolism remains to be established before any relationship between decreased cellular ATP, depressed incorporation of nucleic acid precursors, and mitogen-induced lymphocyte transportation can be established.

In summary, our experiments support the conclusion that valinomycin interferes with cellular function in resting and proliferating lymphocytes as judged by depressed nucleic acid synthesis. The results obtained using valinomycin in intact viable cells in other biological studies should also take into account the side effects of valinomycin on cellular metabolism described in this report.

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A non-α-adrenoceptor binding site for [^{125}I]-BE 2254 in guinea pig brain membranes

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There is currently much interest in the new radioligand ^{125}I-labeled BE 2254* which is proving to be a valuable tool for the study of α_1-adrenoceptors in rat cerebral cortex membranes [1–6]. However, when using [^{125}I]-BE 2254 to study α_1-adrenoceptors in guinea pig cerebral cortex membranes, we found a difference between the total amount of bound [^{125}I]-BE 2254 displaced by saturating concentrations of BE 2254 and phentolamine [7], indicating a second binding site for the radioligand. The present investigation attempts to characterise this second binding site by using displacement studies with drugs which interact with a wide variety of receptors and transport processes and also attempts to establish whether this binding site is present in cerebral cortex membranes from other animal species. Since [^{125}I]-BE 2254 is being used increasingly to characterise α_1-adrenoceptors, it is important to establish the identity and prevalence of this second site. To study the displacement effects of ligands on the second binding site alone, the α-adrenoceptor was first blocked with a saturating concentration of phentolamine.

* Abbreviations: BE 2254, 2-(β-(4-hydroxyphenyl)-ethylaminomethyl)-tetralone; and PMSF, phenylmethylsulfonyl fluoride.

Methods and materials

Radioiodination. BE 2254 was labeled with ^{125}I (Na ^{125}I carrier-free, 100 mCi/ml, from the Radiochemical Center, U.K.) using a modification of the chloramine-T method of Maguire *et al.* [8], and the iodinated product was purified by ethyl acetate extraction followed by paper and thin-layer chromatography as described previously by Adams and Jarrott [7].

Membrane preparation. Cerebral cortex, kidney or seminal vesicle tissue was weighed and homogenised in 50 vol. of 40 mM potassium phosphate buffer, pH 7.4, containing PMSF, centrifuged at 45,000 *g* for 10 min,