BBA 75903

THE EFFECTS OF NIGERICIN, VALINOMYCIN, AND 2,4-DINITROPHENOL ON INTRACELLULAR pH, GLYCOLYSIS, AND K^+ CONCENTRATION OF EHRLICH ASCITES TUMOR CELLS

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(Received November 26th, 1971)

SUMMARY

The effects of nigericin, valinomycin, 2,4-dinitrophenol, and combinations of these drugs on intracellular pH (pH_i), glycolysis, and K^+ concentrations of Ehrlich ascites tumor cells have been studied.

All of the drugs and combinations raised extracellular pH (pH_e) and lowered pH_i in non-glycolyzing cells. All drugs administered with glucose except nigericin increased the rate of glycolysis and produced lower values of pH_e and pH_i than did glucose alone. Nigericin caused some increase in the rate of lactate production with little if any effect on glucose utilization. The values of pH_e and pH_i obtained with glycolyzing cells treated with nigericin alone and in combination with valinomycin indicate effective transfer of H^+ from the external medium into the cells, presumably in exchange for K^+.

All drugs and combinations caused loss of cellular K^+ that was partially inhibited by glucose. The effects of nigericin and valinomycin on K^+ loss were additive. Those of dinitrophenol and valinomycin were not. The effects of dinitrophenol and valinomycin are attributed to depletion of ATP required for Na^+–K^+ membrane transport. An additional effect of nigericin may be promotion of K^+–H^+ exchange across the plasma membrane. The combination of valinomycin *plus* dinitrophenol does not have an effect equivalent to that of nigericin on the plasma membrane.

INTRODUCTION

The antibiotic, nigericin, was first isolated in 1951 from the streptomycete, "Nig-1" (ref. 1). Its effects on respiration, oxidative phosphorylation, and K^+ transport in mitochondria[2–13], ion translocation and photophosphorylation in chloroplasts[14–16], proton movements in chromatophores[17,18], and ion movements across smectic mesophases[10] and the membranes of erythrocytes[4,10,19], submitochondrial particles[2,20], and microsomes[4] have been extensively studied. The effects of nigericin on ion movements across membranes have been interpreted in terms of the formation of a neutral complex between an alkali metal ion and the form of nigericin in which

Abbreviation: DMO, 5,5-dimethyl-2,4-oxazolidinedione.

the carboxyl group is dissociated. Protonation of the carboxyl group releases the cation. Nigericin can catalyze an alkali ion–proton exchange across lipid membranes[4]. By contrast, valinomycin, a molecule lacking an ionizing group, forms positively charged complexes with alkali metal ions and increases the permeability of membranes to those ions but does not permit alkali ion–proton exchange unless an uncoupler of oxidative phosphorylation, which renders the membrane permeable to protons, is also present[4]. The affinities of both nigericin and valinomycin are greater for K^+ than for Na^+ (ref. 4).

Since nigericin had been shown to promote K^+–H^+ exchange in a number of systems studied by other investigators[4,9,16,17,19], it was of interest to ascertain whether this process occurs in the Ehrlich ascites tumor cell and what consequent effects there might be on intracellular pH (pH_i), the pH gradient across the cellular membrane, and glycolytic mechanisms. Since valinomycin *plus* various uncouplers of oxidative phosphorylation have been reported to have similar effects to those of nigericin on ion movements in mitochondria[4], valinomycin and 2,4-dinitrophenol, separately and in combination, have been included for comparison with nigericin in the present study of the Ehrlich tumor cell.

MATERIALS AND METHODS

Nigericin was kindly supplied by Dr J. M. McGuire, Lilly Research Laboratories, Indianapolis, Indiana. Valinomycin was purchased from Calbiochem. The sources of other chemicals and enzymes were previously described[21].

Preparation of cells and incubations

The Ehrlich ascites tumor cells were grown and harvested as previously described[22]. The cells were washed rapidly 4 times in a cold buffer having the composition: NaCl, 0.147 M; KCl, 0.006 M; $MgSO_4$, 0.001 M; Na_2HPO_4, 0.021 M; NaH_2PO_4, 0.004 M. The cells were resuspended in the same buffer at 37 °C under an atmosphere of O_2 for 30 min before addition of drugs or glucose. In those experiments in which pH_i was to be measured, 5,5-dimethyl-2,4-[$^{14}C_2$]oxazolidinedione (DMO) to a concentration of 0.05 μCi (6.5 μg) per ml *plus* [*carboxyl*-^{14}C]inulin to a concentration of about 1 μCi (0.3 mg) per ml were added to the suspensions 15 min before other additions were made. Additions were made and samples were taken as described in the figures and their legends. Additions were made in the following forms: glucose 0.11 M in buffer, 0.1 ml per ml suspension; nigericin (sodium salt) 1 mM in ethanol, 0.01 ml per ml suspension; valinomycin 1 mM in ethanol, 0.01 ml per ml suspension; 2,4-dinitrophenol 3 mM in saline, 0.1 ml per ml suspension. These concentrations of drugs are sufficient to produce maximum effects on both K^+ loss and glycolysis in the intact cell.

Calculation of intracellular pH (pH_i)

The procedure for the use of distribution of [^{14}C]DMO between intracellular and extracellular water for calculation of pH_i in tumor cells *in vitro* has been described in detail in a prevous communication[23].

Analytical methods

The enzymatic methods for glucose, lactate, and glycolytic intermediates have been cited in earlier publications[21,13].

Intracellular K^+ was determined by flame photometry on a 0.1 M HNO_3 digest of a centrifuged cellular pellet with correction for the K^+ trapped in the extracellular space[24].

RESULTS

Intracellular pH (pH_i) and extracellular pH (pH_e)

Fig. 1 shows representative experiments in which measurements of pH_e and pH_i were made in suspensions of Ehrlich ascites tumor cells treated with nigericin, valinomycin, dinitrophenol, and combinations in the absence of glucose. All of the treatments caused pH_e to rise above control levels. At 15 min, all treatments had caused falls of pH_i; but at 30 min, the pH_i of cells treated with nigericin, valinomycin, and a combination of the two had returned to or near control values. Only in cells treated with dinitrophenol and dinitrophenol *plus* valinomycin did pH_i remain low at 30 min.

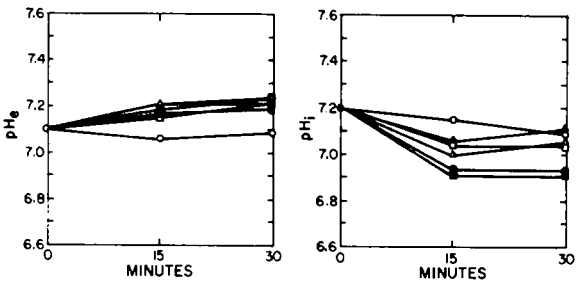

Fig. 1. Extracellular pH (pH_e) and corresponding intracellular pH (pH_i) values in suspensions of Ehrlich ascites tumor cells (approx. 20% by vol.) in 25 mM Krebs–Ringer phosphate buffer. Drugs or combinations of drugs were added at time zero. In these experiments, no glucose was added. ○, no drug; △, nigericin 10 μM; □, valinomycin 10 mM; ●, 2,4-dinitrophenol 0.3 mM; ▲, nigericin 10 μM *plus* valinomycin 10 μM; ■, 2,4-dinitrophenol 0.3 mM *plus* valinomycin 10 μM.

In the presence of glucose, as shown in Figs 2 and 3, all the drugs except nigericin caused both pH_e and pH_i to fall below control levels. Nigericin caused less fall in pH_e and pH_i than any of the other drugs or combinations. In six of eight experiments with glycolyzing cells, somewhat lower values of pH_e were attained when nigericin was present in the medium. In the experiment of Fig. 2, nigericin had no significant effect on pH_e but did result in lower values of pH_i. Nigericin in combination with valinomycin caused less fall of pH_e and greater fall of pH_i than did valinomycin alone. In all experiments in which dinitrophenol was compared directly with any of the other drugs in the same cell population, the greatest falls of both pH_e and pH_i were produced by dinitrophenol. Addition of valinomycin to dinitrophenol decreased the rate of fall of pH_e produced by dinitrophenol and in most cases also decreased slightly the effect of that drug on pH_i.

Glucose utilization, lactate production and glycolytic intermediates

As shown in Fig. 2, which is typical of five experiments, nigericin produced some increase in the rate of lactate production with little if any effect on glucose utilization. Valinomycin had a greater effect than nigericin on lactate production.

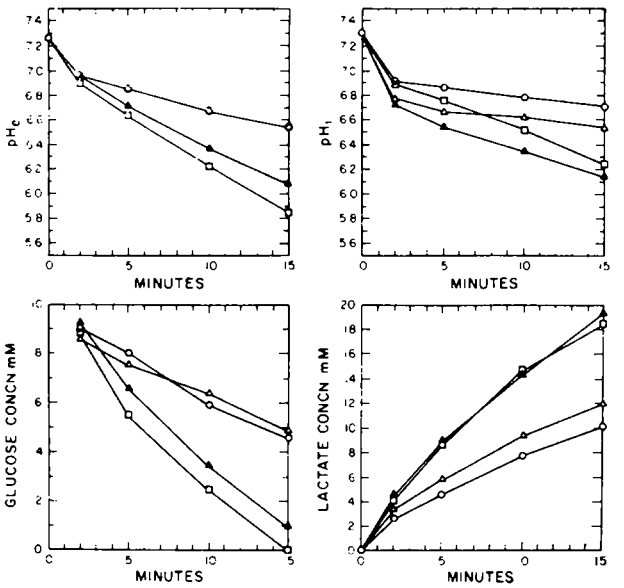

Fig. 2. Extracellular pH, intracellular pH, and glucose and lactate concentrations in the extracellular phase in suspensions of Ehrlich ascites tumor cells (approx. 18% by vol.). The experiments of this figure were performed at the same time on portions from the same cell suspension. Glucose to a final concentration of 11 mM was added at time zero to all suspensions. Drugs were also added at time zero. ○, glucose alone; △, nigericin 10 μM; □, valinomycin 10 μM; ▲ nigericin 10 μM *plus* valinomycin 10 μM.

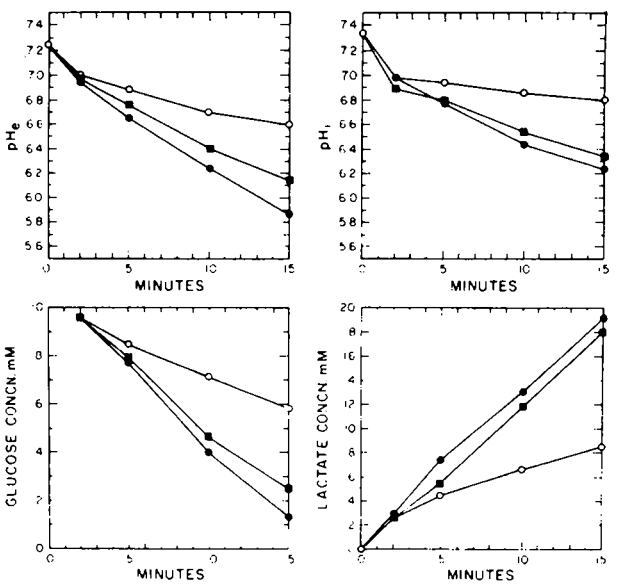

Fig. 3. Experiments performed in the same manner as those of Fig. 2 on suspensions of Ehrlich ascites tumor cells (approx. 15% by vol.). ○, glucose alone; ●, 2,4-dinitrophenol 0.3 mM; ■, 2,4-dinitrophenol 0.3 mM *plus* valinomycin 10 μM.

In all experiments in which valinomycin and dinitrophenol were compared directly in cells from the same preparation, the effects of dinitrophenol on glucose utilization and lactate production were greater than those of valinomycin. Addition of valinomycin to dinitrophenol actually decreased these effects slightly.

The sum of the glycolytic intermediates, fructose 1,6-diphosphate and dihydroxyacetone phosphate (principally the latter), shown in Fig. 4, was reduced relative to the control value by all drug treatments. Glyceraldehyde 3-phosphate, which was also determined, was negligible in all samples. Although valinomycin and dinitrophenol both have similar effects in increasing glucose utilization, valinomycin lowers

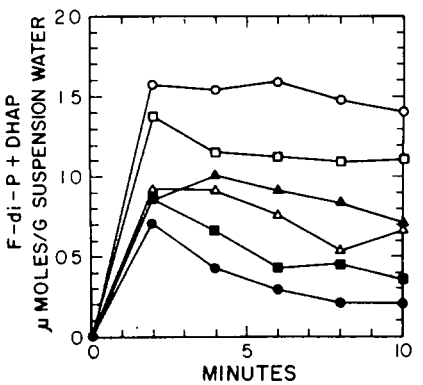

Fig. 4. Sum of concentrations of the glycolytic intermediates, fructose 1,6-diphosphate (F-di-P) and dihydroxyacetone phosphate (DHAP) (principally the latter), in suspensions of Ehrlich ascites tumor cells. Concentrations of glyceraldehyde 3-phosphate were negligible in all samples. The values shown are averages from four experiments. Symbols, drug concentrations, and conditions of the experiments are the same as those of Figs 2 and 3.

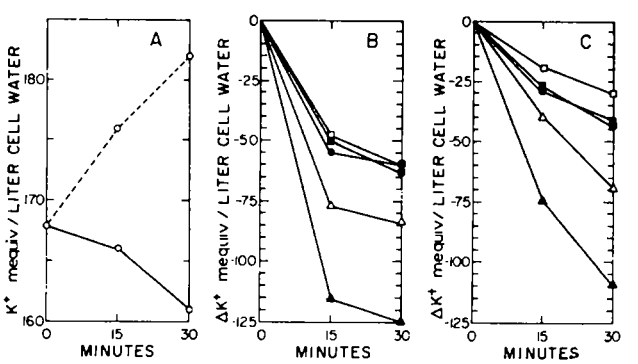

Fig. 5. Intracellular content of K^+ in Ehrlich ascites tumor cells suspended in Krebs–Ringer phosphate buffer. In A, no drugs were added. ———, no glucose; – – – –, glucose to a concentration of 11 mM added at time zero. Each point represents the average of 10 experiments. In B and C, drugs or combinations of drugs were added at time zero. In B, no glucose was added. In C, glucose to a concentration of 11 mM was added at time zero together with the drugs. Values shown in B and C are differences between K^+ concentrations in cells treated with a drug and those in corresponding untreated control cells. Each point plotted is the average of these differences in several experiments (number to be given in parentheses). △, nigericin 10 μM (8 in B, 9 in C); □, valinomycin 10 μM (6 in B, 8 in C); ●, 2,4-dinitrophenol 0.3 mM (10 in B, 10 in C); ▲, nigericin 10 μM *plus* valinomycin 10 μM (10 in B, 10 in C); ■, 2,4-dinitrophenol 0.3 mM *plus* valinomycin 10 μM (7 in B and C).

the levels of intermediates much less than does dinitrophenol. Nigericin lowers the intermediates more than does valinomycin and less than does dinitrophenol.

Cellular K^+

Concentrations of intracellular K^+ are shown in Fig. 5. Chart A shows that in the absence of drugs there was a slight loss of K^+ from the cells during the incubation period when there was no glucose and a more pronounced uptake of K^+ when glucose was present. In Chart B, it is seen that all of the drugs and combinations caused loss of intracellular K^+ in the absence of glucose. As shown in Chart C, the loss of K^+ with all drug treatments was partially inhibited by glucose. Four additional experiments in which the K^+ loss from glycolyzing cells as affected by drugs was determined at 2, 5 and 10 min showed no essential difference from the pattern of Chart C of Fig. 5. In the presence or absence of glucose, the effects of valinomycin and dinitrophenol were not additive. The effect of nigericin was greater than that of any of the other drugs, and the effects of nigericin in combination with valinomycin were additive.

DISCUSSION

In the absence of glucose, all of the drugs and combinations used here caused rises of pH_e and falls of pH_i. All of the drugs also caused loss of intracellular K^+. However, in no case can the changes of pH_e and pH_i be accounted for as simple 1:1 exchanges of K^+ for H^+. The increases of extracellular K^+ concentrations were much larger than the calculated amounts of H^+ removed from the medium. The large and persistent fall of pH_i seen in the presence of dinitrophenol alone and with valinomycin may be attributed in part to hydrolysis of ATP.

As reported in earlier studies[23,24], the values of pH_e found after administration of glucose alone or glucose with valinomycin are in agreement with those calculated for the addition of the observed extracellular concentrations of lactic acid to the buffer, and the relationship of pH_i to pH_e is about the same as in the non-glycolyzing cells.

In the present experiments, as exemplified in Fig. 3, the pH gradient across the plasma membrane was not abolished by dinitrophenol as it was in experiments previously reported[25]. The glucose concentrations in the experiments now being reported were twice those previously used. This resulted in higher lactate concentrations and corresponding lower values of pH_e. There appears to be a limit below which pH_i cannot be forced by glycolysis.

In all of five experiments with glycolyzing cells treated with nigericin alone and in three with cells treated with nigericin combined with valinomycin, the measured values of pH_e were significantly higher than the values calculated from the lactate concentrations. Furthermore, the values of pH_i in these cells were lower than those found in the present and previous experiments for corresponding values of pH_e in cell suspensions exposed to glucose alone[23,24]. This is indicative of effective transfer of H^+ from the external medium into the cells. When comparison is made between the concentrations of lactic acid calculated to have been effectively neutralized in the external medium and the concentrations of K^+ added to that medium, the latter are larger but not of a different order of magnitude. For instance, in one experiment with nigericin alone, 1.5 mM of the lactate in the medium at 15 min was apparently

neutralized, and extracellular K^+ had increased by 5.2 mM. In an experiment with nigericin *plus* valinomycin, the lactate in the medium at 15 min was neutralized to the extent of 4.6 mM, and 8.6 mmoles of K^+ per l had been added to the medium from the cells. The sensitivity of these measurements is not, however, such as to support or refute the inference that the pH relationships are explicable entirely in terms of 1:1 K^+–H^+ exchange across the plasma membrane.

The apparent transfer of H^+ from the external medium into the cells brought about by nigericin decreases the pH_e–pH_i gradient. In several experiments, including that of Fig. 2, this gradient had almost entirely disappeared at 10 and 15 min. Valinomycin increases the pH gradient above control values. In each of five experiments in which the effects of valinomycin alone and of valinomycin *plus* nigericin were compared in portions of the same cell preparations, the presence of nigericin resulted in a lower pH gradient than that found with valinomycin alone.

The slight effect that nigericin had in stimulating glycolysis may be due to a stimulation of mitochondrial ATPase[2] caused by the high concentrations used in these experiments. In lower concentrations nigericin has been reported not to uncouple oxidative phosphorylation[3]. The stimulation of glycolysis produced by valinomycin can be attributed at least in part to its uncoupling effect and consequent increased levels of ADP. Dinitrophenol in the concentration used here appears to have a maximal effect on glycolysis. Addition of valinomycin to dinitrophenol does not increase the rate of glycolysis.

As we have previously reported[24], valinomycin causes loss of intracellular K^+. We have attributed this effect to depletion of cellular ATP required for the energy-consuming transport system responsible for maintaining high cellular K^+ and low cellular Na^+. This is in accord with the earlier suggestion of Levinson[26] to account for the inhibition by valinomycin of the uptake of K^+ and loss of Na^+ in K^+-depleted cells. The partial reversal by glucose of the K^+ loss produced by valinomycin may represent the utilization for cation transport of the ATP generated by glycolysis.

The fact that the effect of dinitrophenol on K^+ loss is nearly the same as that of valinomycin and is not augmented by addition of valinomycin is indicative that both drugs act on the plasma membrane through the same ultimate mechanism of ATP depletion and that each produces nearly a maximal effect on that mechanism. This depletion of ATP may be brought about by the two drugs by entirely different mechanisms in the mitochondrion.

If valinomycin and dinitrophenol are producing the maximal effect on K^+ loss that can be attained by inhibition of the energy-dependent membrane Na^+–K^+ transport system, it follows that the greater loss of K^+ induced by nigericin is brought about through another mechanism. The additive effect on K^+ loss obtained by combination of valinomycin with nigericin is in accord with this concept. Inhibition of the Na^+–K^+ membrane transport system by valinomycin would allow movement of those ions down their concentration gradients. If nigericin promotes a K^+–H^+ exchange across the plasma membrane, this would provide an additional mechanism for passage of K^+ out of the cell. The possibility is not excluded of nigericin also acting in part by depletion of ATP. This could explain the partial reversal of the nigericin effect by glucose.

If glucose should enter the cells together with Na^+ by a coupled transport system, as has been postulated for cells where glucose is transported against a con-

centration gradient[27], the consequent extrusion of Na^+ by activation of the Na^+–K^+ membrane pump would decrease the apparent K^+ loss. This represents an additional conceivable mechanism by which glucose could decrease K^+ loss effected by any of the drugs and drug combinations.

The transport in opposite directions of K^+ and H^+ by nigericin appears to be operative in the plasma membrane of the Ehrlich ascites tumor cell as it has been reported to be by other investigators in a number of other membrane systems. However, an equivalent effect on the plasma membrane of this cell is not produced by a combination of valinomycin and an uncoupler of oxidative phosphorylation, as has been reported for mitochondria.

ACKNOWLEDGEMENTS

This investigation was supported by U. S. Public Health Service Research Grant No. GM 13606 and U. S. Public Health Service Career Program Award 4 K06 GM 19429 for T. C. B. from the National Institute of General Medical Sciences and by Grant P-603 from the American Cancer Society.

We wish to thank Hollis Hawkins for expert technical assistance.

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