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K^+/H^+-antiporter nigericin arrests DNA synthesis in Ehrlich ascites carcinoma cells

(intracellular pH/cell proliferation/cytostatics)

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ABSTRACT **Acidification of the cytoplasm of Ehrlich ascites carcinoma cells to pH 6.3 arrests DNA synthesis in these cells. Such an effect can be achieved by incubating the cells at pH 6.2 or by adding low concentrations of the K^+/H^+ antiporter, the antibiotic nigericin, at neutral pH. Glucose and anaerobiosis potentiate the nigericin effect. The inhibition of DNA synthesis by nigericin occurs without any significant decrease in the ATP concentration and in the mitochondrial membrane potential. The DNA synthesis inhibition is caused neither by a decrease in the intracellular $[K^+]$ nor by an increase in the intracellular $[Na^+]$ accompanying the nigericin effect (at least at low concentrations of the antibiotic). Nigericin should thus be regarded as a type of a cytostatic primarily affecting intracellular pH.**

Several studies have shown a strong dependence of the rate of DNA synthesis in animal cells upon the intracellular pH (pH_i) level (1–4). It was demonstrated that initiation of DNA synthesis in stimulated fibroblasts and lymphocytes requires a cytoplasmic pH shift to the alkaline region (2). This shift proved to be as small as 0.2 pH unit—namely, from 7.2 (no DNA synthesis) to 7.4 (maximal rate of the synthesis).

Such a steep pH–response relationship (in fact, of the all-or-nothing type) suggests that we deal here with a specific regulatory event rather than with a trivial pH dependence inherent in any enzymatic system. Further indication in this direction was obtained when it was revealed that the phosphatidylinositol cascade and the Na^+/H^+ antiporter are involved in the above-mentioned pH_i increase. It was proposed that H^+ ions, like Ca^{2+}, cyclic nucleotides, and inositol phosphates, serve as second (intracellular) messengers of extracellular signals (2, 5).

The Na^+/H^+ antiporter appears to be one of the most important mechanisms for maintaining a sufficiently high pH_i in mammalian cells (1, 3, 6, 7). It extrudes H^+ from the cell in exchange for extracellular Na^+, using the Na^+,K^+-ATPase-produced Na^+ gradient as a driving force. As to the K^+ gradient, also formed by Na^+,K^+-ATPase, it is not involved in pH regulation. In the present study we have tried to employ the K^+ gradient to return extruded H^+ ions to the cytoplasm. In this way, one may hope to overcome the pH_i increase and so to stop DNA synthesis, using the same energy source—i.e., Na^+,K^+-ATPase. To do this, it seems necessary to initiate a K^+/H^+ antiport in the plasma membrane. The first indication that the exogenous K^+/H^+ antiporter, nigericin, is inhibitory for DNA synthesis in the animal cell was obtained in this group, when ascites cells were studied (8, 9). Then Rotin *et al.* (10) reported the cytotoxic effect of nigericin on ovary and bladder cancer cells. However, this group reported later that, due to long-term incubation, nigericin and amiloride could affect the cell proliferation in a nonspecific way, rather than by pH_i decrease (11). In this paper, we shall show that the mechanism of the nigericin action on the Ehrlich carcinoma cell consists of the pH_i shift below the threshold level permitting DNA synthesis.

MATERIALS AND METHODS

Cells. Ehrlich ascites tumor cells were used on the fifth to seventh day after i.p. implantation into white mice. During experiments, 3×10^7 cells per ml were incubated at 37°C in 199 medium buffered with 50 mM Tris·HCl (pH 7.0–7.8) or 50 mM Mes-NaOH (pH 6.2–6.9). The incubation mixture was continuously shaken. In the anaerobic experiments, argon barbotage was employed.

Reagents. The following reagents were used: nigericin (Calbiochem), thiamine (Serva), fluorescein diacetate (FDA) (Fluka), 2′,7′-bis(2-carboxyethyl)-5-(and-6)carboxyfluorescein (acetoxymethyl ester) (BCECF-AM) (Molecular Probes), [^{14}C]thymidine (Isotope, USSR), glucose (Reanal, Budapest), choline chloride (Serva), rhodamine 123 (Sigma), Tris (Sigma), and Mes (Serva).

Measurement of the DNA Synthesis. [^{14}C]Thymidine was added to the incubation mixture 15 min after the addition of the cells. Aliquots of the cell suspension were sedimented immediately, 15 or 30 min after the [^{14}C]thymidine addition. The radioactivity of the acid-insoluble fraction of these cells was evaluated in a Mark III liquid scintillation counter (Nuclear-Chicago).

Measurement of pH_i. The pH_i was evaluated by using a pH-sensitive dye, FDA or BCECF-AM (12). The cell suspension was stained for 10 min with 0.01 mM FDA or 3 μM BCECF-AM. The excess dye was removed by washing the cells. The ratio of fluorescence intensity at two wavelengths (430 and 490 nm) was measured with an Opton fluorescence microscope (Zeiss) equipped with an FML-2 microfluorimeter (LOMO, Leningrad, USSR). To estimate the pH_i value, the nigericin-K^+ method was employed (13).

Measurement of Intracellular Na^+ and K^+ Levels. After incubation, cells were washed twice with a 10-fold (vol/vol) excess of 0.14 M choline chloride. The final cell sediment was frozen, thawed, and suspended in distilled water. Then the Na^+ and K^+ concentrations were measured with a flame photometer.

Measurement of ATP. After incubation, the cells were sedimented by centrifugation, and ATP was extracted from the sediment with trichloroacetic acid at 0°C. To measure ATP in the extract, the luciferin-luciferase system and Pico-ATP luminometer (Jobin-Yvon, Longjumeau, France) were used (for details, see ref. 14).

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Abbreviations: BCECF-AM, 2′,7′-bis(2-carboxyethyl)-5-(and-6)carboxyfluorescein (acetoxymethyl ester); FDA, fluorescein diacetate; subscripts "i" and "o" (e.g., pH_i and pH_o), intracellular and outer (extracellular) compartments.

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Table 1. Dependence of DNA synthesis on pH_i

pH_o	7.8	7.5	6.9	6.5	6.25
pH_i*	7.1 ± 0.02	6.9 ± 0.02	6.6 ± 0.04	6.4 ± 0.03	6.3 ± 0.02
% DNA synthesis*	100 ± 9	100 ± 6	100	57 ± 2	8 ± 2

To vary pH_i, the pH_o values were changed. DNA synthesis was measured by [^{14}C]thymidine incorporation into DNA. The rate of [^{14}C]thymidine incorporation at pH_o equal to that in ascitic liquid (6.9) was taken as 100%.
*Values are given as mean ± SEM.

Mitochondrial Membrane Potential Probe. As a mitochondrial membrane potential probe, we employed the penetrating fluorescing cation rhodamine 123 (15, 16). The rhodamine staining of mitochondria in the living cell was observed with an Opton fluorescence microscope. The rhodamine concentration was 0.01 mg/ml.

RESULTS AND DISCUSSION

pH_i Dependence of DNA Synthesis in Ascites Cells. The dependence of DNA synthesis in ascites cells on pH_i is presented in Table 1. Different pH_i values were obtained by varying the pH of the incubation medium (pH_o). The ascites cells [like the activated fibroblasts studied earlier (2)] show a very steep pH_i–DNA synthesis relationship so that the transition from the maximal synthesis to its almost complete inhibition occurs with the pH_i decrease by 0.3. However, compared with fibroblasts, the curve for Ehrlich ascites cells is shifted to the low pH region. The half-maximal inhibition in these cells takes place at pH 6.4 [compare with pH = 7.3 in fibroblasts (2)]. Apparently, such a shift is the result of adaptation of the tumor cell to the acidic conditions. The permanently increased activity of the Na^+/H^+ antiporter could be the mechanism of this adaptation (17, 18).

Effects of Nigericin on pH_i and DNA Synthesis. The gradients of H^+ and K^+ across the ascites cell plasma membrane are unidirectional—namely, higher [H^+] and [K^+] inside than outside. Under physiological conditions, the K^+ gradient is almost two orders higher than the H^+ gradient. Thus the K^+/H^+-antiporter nigericin should import H^+ into the cytoplasm and export K^+ out of the cell. The measurements of pH_i (Table 2) and $[K^+]_i$ (see below, Fig. 2) showed that this was the case. As seen from Table 2, pH_i in the nigericin-treated cells is ≈0.3 unit lower than that in the nontreated cells. Such a pH_i decrease was found to inhibit the DNA synthesis when the pH_i value fell below the threshold level (6.4) (Fig. 1).

A further decrease in the effective nigericin concentration (by about one order of magnitude) was achieved when the incubation was carried out in the presence of glucose under anaerobic conditions. Almost complete inhibition of the DNA synthesis was observed at 0.5 μM nigericin at pH_o = 7.0. Without nigericin, glucose and anaerobiosis were practically without any measurable effect (Table 3).

Control experiments showed that the above described effects of nigericin on [^{14}C]thymidine incorporation into DNA were not due to changes in thymidine transport across the cell plasma membrane.

Effect of pH_o Decrease and of Nigericin on $[K^+]_i$, $[Na^+]_i$, $[ATP]_i$, and Energization of Mitochondria. Fig. 2 shows measurements of DNA synthesis and intracellular K^+ concentration in ascites cells under various conditions. The inhibition of DNA synthesis by lowering of pH_o was found to

Table 2. pH_i of nigericin-treated Ehrlich ascites carcinoma cells

	pH_i	
pH_o	Control	0.01 mM nigericin
6.9	6.6 ± 0.05	6.3 ± 0.07
7.8	6.9 ± 0.06	6.6 ± 0.05

Values are given as mean ± SEM.

Table 3. Potentiation of the nigericin-induced inhibition of DNA synthesis by glucose and anaerobiosis at pH_o = 7.0

	[^{14}C]Thymidine incorporation, %		
	Aerobiosis		Anaerobiosis
Addition	Without glucose	20 mM glucose	(20 mM glucose)
None	100	90 ± 10	100 ± 1
0.5 μM nigericin	90 ± 5	50 ± 5	20 ± 15

The level of [^{14}C]thymidine incorporation under aerobic conditions without nigericin and glucose was taken as 100%. Values are given as mean ± SEM.

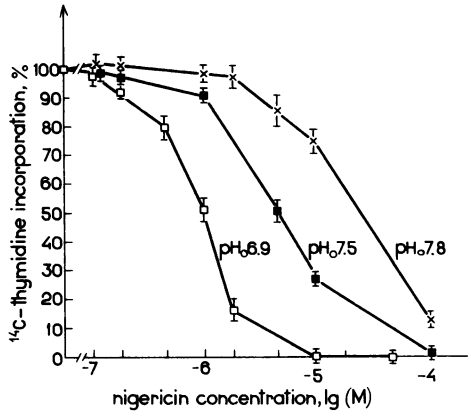

FIG. 1. Inhibition of DNA synthesis in ascites cells by nigericin at various pH_o values. [^{14}C]Thymidine incorporation at pH 6.9 was taken as 100%.

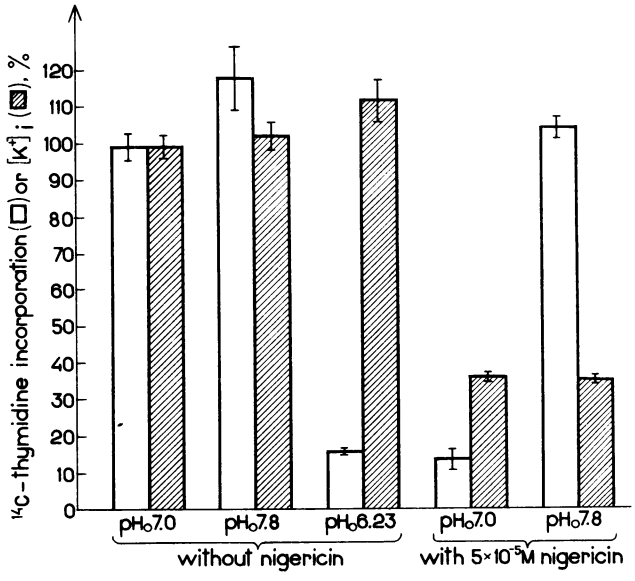

FIG. 2. Comparison of the nigericin effects on DNA synthesis and intracellular K^+ concentration in ascites cells. [^{14}C]Thymidine incorporation and $[K^+]_i$ at pH_o = 7.0 were taken as 100%.

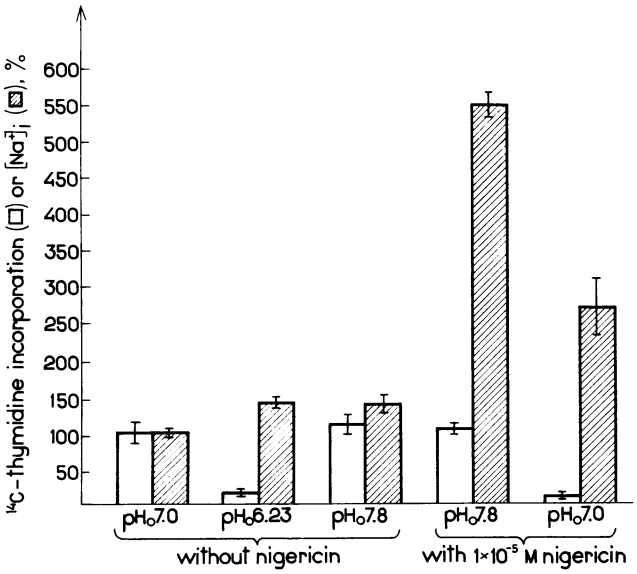

FIG. 3. Comparison of the nigericin effects on DNA synthesis and intracellular Na^+ concentration in ascites cells. [^{14}C]Thymidine incorporation and $[Na^+]_i$ at pH_o = 7.0 were taken as 100%.

develop without any significant changes in $[K^+]_i$. On the other hand, nigericin strongly decreased $[K^+]_i$ at pH_o = 7.0, when it arrested the DNA synthesis, as well as at pH_o = 7.8, when nigericin-induced inhibition of the DNA synthesis was absent.

According to Fig. 3, acidification of the outer medium inhibited DNA synthesis with no $[Na^+]_i$ changes observed. As to nigericin, it increased $[Na^+]_i$ at pH_o = 7.0 (DNA synthesis is inhibited) and at pH_o = 7.8 (no DNA synthesis inhibition).

It is clear from Fig. 4 that the lowering of the DNA synthesis rate by nigericin occurred without any dramatic changes in the level of the intracellular ATP. Under conditions of almost complete inhibition of thymidine incorporation in the presence of 5 μM nigericin, ATP was maintained at a level equal to 85% of that without nigericin.

Finally, the possible effect of nigericin on mitochondria was tested. Mitochondria in ascites cells were stained with the cationic fluorescent penetrant, rhodamine 123, which is known to be electrophoretically accumulated by mitochondria.

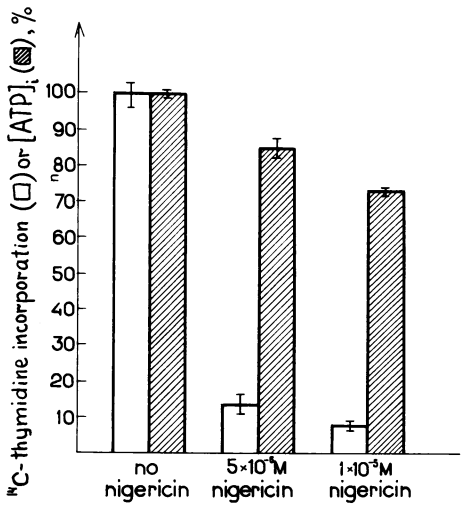

FIG. 4. Comparison of the nigericin effects on DNA synthesis and ATP level in ascites cells. [^{14}C]Thymidine incorporation and $[ATP]_i$ at pH_o = 7.0 were taken as 100%.

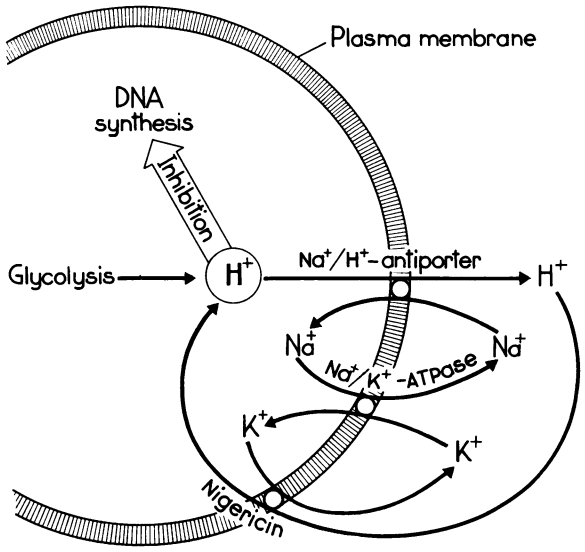

FIG. 5. Tentative scheme illustrating interplay of glycolysis, Na^+,K^+-ATPase, Na^+/H^+ antiporter, and nigericin in the ascites cell.

It was found that nigericin concentrations strongly inhibiting the DNA synthesis did not change the fluorescence of mitochondria inside the ascites cells (not shown). Thus under conditions used, nigericin did not affect the mitochondrial membrane potential.

Thus, although the effect of nigericin is accompanied by a $[K^+]_i$ decrease, by a $[Na^+]_i$ increase, and by a small change in the ATP level, these effects are hardly responsible for the observed inhibition of the [^{14}C]thymidine incorporation rate (at low nigericin concentrations at least). It should be mentioned in this context that Rotin *et al.* (10) observed a cytotoxic effect of nigericin on ovary and bladder cancer cells, which was accompanied by a pronounced decrease in the ATP level. This was apparently due to a rather long (4 hr) period of nigericin treatment (15 min in our experiments). It is interesting that Rotin and coworkers (10) reported that anaerobiosis somehow sensitizes the cells to the cytotoxic action of low nigericin concentrations. A similar effect was observed in our study. It was especially conspicuous if glucose was present (Table 3).

Thus, the above data show that the cytoplasm acidification caused either by the pH_o decrease or by the nigericin-mediated H^+_o/K^+_i antiport results in the inhibition of DNA synthesis in ascites tumor cells. The effect of nigericin is potentiated by conditions favorable for activation of glycolysis (anaerobiosis, high glucose level). Perhaps it is the pH_i decrease that is responsible for the favorable therapeutic action of hyperglycemia in certain cases of cancer (10). One may hope that the combined use of nigericin and hyperglycemia will affect tumor cells rather than the normal ones in which glycolysis and hence the production of H^+ ions are usually much slower (5). It is not surprising therefore that—e.g., in thymic lymphocytes—nigericin did not inhibit the early response to mitogens—i.e., increase in the level of mRNA of c-*fos* protooncoprotein (11).

The interplay of glycolytic H^+ production, Na^+/H^+ antiporter, Na^+,K^+-ATPase, and nigericin is summarized in Fig. 5.

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