Nigericin-induced Death of an Acidophilic Bacterium

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(Received 20 November 1978; revised 5 February 1979)

At an external pH of 3.5, nigericin (which catalyses an electroneutral H^+/K^+ exchange) abolished the transmembrane proton gradient (ΔpH) of *Bacillus acidocaldarius*, causing a rapid acidification of the cytoplasm from approximately pH 6.0 to pH 3.5. A pronounced loss of viability and fine-structural changes rapidly followed treatment with nigericin. A marked decline in respiration and an even more rapid decrease in cytoplasmic ATP were observed. Activity of at least one cytoplasmic enzyme decreased more slowly. There was no generalized loss in the integrity of the cytoplasmic membrane, as assayed by permeability to inulin or Na^+ or by release of ultraviolet light-absorbing compounds. The loss of viability upon treatment with carbonyl cyanide *m*-chlorophenylhydrazone was similar to that observed with nigericin, so proton influx alone, rather than together with K^+ efflux, was probably involved in the death of the organism. Moreover, acidification of the cytoplasm rather than abolition of the ΔpH was the lethal event, since no loss of viability was observed when the ΔpH was abolished by elevation of the external pH.

INTRODUCTION

In accord with Mitchell's chemiosmotic hypothesis (1961, 1963), bacteria generate, via respiration and/or ATP hydrolysis, a protonmotive force generally consisting of a transmembrane pH gradient (ΔpH , exterior acid) and a transmembrane electrical potential ($\Delta\psi$, exterior positive). The protonmotive force, in turn, energizes a variety of energy-requiring processes, as reviewed by Harold (1977). When we became interested in the protonmotive force in acidophilic bacteria, it was with the expectation that such organisms would maintain extremely large pH gradients across the membrane. Thus, bacteria which could grow at external pH values of 2 to 3 would presumably require cytoplasmic pH values much closer to neutrality in order to maintain viability. The consistently large ΔpH resulting from this requirement would, moreover, probably preclude the existence of any appreciable $\Delta\psi$, exterior positive. In fact, the occurrence of a 'reversed' $\Delta\psi$, i.e. interior positive, seemed a likely adaptation in such organisms.

Our own studies with *Bacillus acidocaldarius* (Krulwich *et al.*, 1978) and those of others with different acidophiles (Hsung & Haug, 1975, 1977; Searcy, 1976) have indeed shown that such organisms maintain a cytoplasmic pH of greater than 5, thus generating large ΔpH values, and concomitantly exhibiting reversed $\Delta\psi$ values. The assumption that organisms would need to maintain cytoplasmic pH values much closer to neutrality than a highly acidic milieu appeared validated. Thus, if the large ΔpH in an acidophile such as *B. acidocaldarius* were abolished so that rapid acidification of the cytoplasm occurred, viability of the organism would be threatened. In the study presented here, nigericin was used to abolish the ΔpH in *B. acidocaldarius* and the ensuing death of the organism was monitored and characterized. The results indicated that the constraints imposed by a lower cytoplasmic pH limit upon viability are quite severe.

METHODS

Organism and growth conditions. *Bacillus acidocaldarius* ATCC 27009 was grown with shaking at 50 °C in a basal salts medium adjusted to pH 3.5 (Krulwich *et al.*, 1978). Lactose (25 mM) was added from a separate sterile solution whose pH was adjusted to 3.5. Solid medium contained the same salt solution and lactose, with the addition of 1% (w/v) purified agar. The agar was autoclaved separately and mixed with the salt solution at 50 °C, after which Petri plates were poured.

Chemicals. [*carboxy*-¹⁴C]Acetylsalicylic acid, [³H]inulin (100 mCi g⁻¹), ²²NaCl (carrier-free) were purchased from New England Nuclear. Potassium [¹⁴C]thiocyanate (40 mCi mmol⁻¹) was from The Radiochemical Centre, Amersham. Firefly tails, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), ATP (disodium salt) and *o*-nitrophenyl- β -D-galactopyranoside were from Sigma. Nigericin was the generous gift of Dr H. R. Kaback. All other chemicals were obtained commercially at the highest purity available.

Treatment with nigericin. Exponentially growing organisms were washed twice at room temperature with 25 mM-sodium citrate buffer, pH 3.5, and suspended in that buffer to give 0.05 mg bacterial protein ml⁻¹. The suspensions were incubated with shaking at 50 °C for 10 min. Nigericin (in ethanol) was then added to a final concentration of 0.2 μ M, and samples were taken at intervals. For measurements of viability, the nigericin-treated organisms were rapidly diluted (1 in 10⁴) in 25 mM-sodium citrate, pH 3.5, and plated on solid media. For measurements of ATP, oxygen consumption, β -galactosidase (β -D-galactoside galactohydrolase; EC 3.2.1.23) activity and uptake of various solutes, the samples were filtered through Millipore type HA filters (0.45 μ m pore size) and washed with 25 mM-sodium citrate, pH 3.5. Organisms were collected from the filters, suspended in citrate buffer and used for the appropriate assay.

Addition of 0.2 μ M-nigericin, under the conditions described, totally dissipated the transmembrane Δ pH within 5 s (Krulwich *et al.*, 1978). Measurements of the pH of the medium indicated that the pH of the suspension increased by less than 0.02 pH units. Thus, the nigericin treatment rapidly reduced the cytoplasmic pH from approximately 6.0 to 3.5. On the other hand, measurements of the transmembrane $\Delta\psi$ indicated no change during the nigericin treatment from a value of 35 mV (interior positive).

Every experiment with nigericin included a control in which organisms were incubated under the same conditions in the absence of nigericin. All the data represent average values from no fewer than three separate experiments.

Assays. Cytoplasmic ATP was extracted with HClO₄ (Cole *et al.*, 1967) and measured by the firefly assay (Stanley & Williams, 1969) in a Beckman LS-230 spectrometer, as described elsewhere (Krulwich *et al.*, 1978). Determinations of oxygen consumption were made by placing 3 ml of bacterial suspension in a Yellow Springs Instruments model J3 oxygen monitor attached to a Beckman chart recorder. Lactose was added to a final concentration of 3 mM, and the suspensions were incubated with stirring in the measuring chamber for 5 min. Oxygen uptake was monitored for 5 to 10 min at 50 °C. β -Galactosidase was assayed according to the method of Craven *et al.* (1965). The proton gradient across the membrane (Δ pH) was measured by the distribution of [*carboxy*-¹⁴C]acetylsalicylic acid (55 μ M, 125 μ Ci mg⁻¹) at 50 °C in a flow dialysis apparatus (Ramos *et al.*, 1976) as described elsewhere (Krulwich *et al.*, 1978). The transmembrane electrical potential ($\Delta\psi$) was measured as described previously (Krulwich *et al.*, 1978) by the uptake of KS¹⁴CN (2.5 mM, 0.25 Ci mmol⁻¹). The uptake of ²²NaCl or [³H]inulin was measured using a filtration assay at 50 °C as described previously for methyl thio- β -D-galactoside uptake (Krulwich *et al.*, 1978). Protein was determined by the method of Lowry, using lysozyme as the standard.

Electron microscopy. Organisms were fixed by the method of Kellenberger *et al.* (1958), dehydrated in a graded series of alcohols and embedded in Epon 812 (Luft, 1961). Sections were cut on a Porter-Blum MT-2 ultramicrotome with a diamond knife and stained with uranyl acetate and lead citrate (Reynolds, 1963). The specimens were examined in a JEOL 100 electron microscope at 80 kV.

RESULTS AND DISCUSSION

Bacillus acidocaldarius was treated with nigericin under conditions in which, as described in Methods, the transmembrane Δ pH was abolished and the cytoplasm was rapidly acidified to pH 3.5. This treatment resulted in death of the organisms (Fig. 1). More than 50% of the colony-forming capacity was lost after 5 min nigericin treatment. Organisms incubated under similar conditions in the absence of nigericin exhibited a definite, though much smaller and more variable, loss of viability over 2 h. Examination of thin sections by electron microscopy (Fig. 2) revealed that *B. acidocaldarius* has morphological features typical of bacilli, but after 2 min nigericin treatment there was a marked change in the

Fig. 1. Effect of nigericin on the viability of *B. acidocaldarius*. Organisms suspended in 25 mM-sodium citrate, pH 3.5, were incubated at 50 °C in the absence (○) or presence (●) of 0.2 μ M-nigericin. Samples were taken at the indicated times to determine the number of colony-forming units.

appearance of the cytoplasm. Septa, mesosomes, a nuclear region and a rather homogeneous cytoplasm were all discernible in control cultures (Fig. 2*a*). After 2 min nigericin treatment, a nuclear region and mesosomes were no longer discernible and the cytoplasm appeared to contain aggregates of particulate material which previously had been homogeneously distributed (Fig. 2*b*). Septa were still observed in some organisms, and the cytoplasmic membrane and walls appeared intact but separated by a distinct gap. Incubation of control and nigericin-treated cultures for another 30 min did not cause any further changes in morphology (results not shown).

Respiratory activity and cytoplasmic ATP content declined rapidly to very low levels on treatment with nigericin (Figs 3, 4). The decline in ATP was especially rapid, further experiments showing it to be complete within 1 min after the addition of nigericin (results not shown). An appreciable, though much slower, decline in ATP occurred in control organisms incubated in the non-nutrient buffer in the absence of nigericin (Fig. 4). Presumably there is a gradual loss of the Δ pH with concomitant acidification of the cytoplasm in the control organisms, although this was not examined. The ATP lost from treated and untreated organisms did not appear in the medium. Perhaps acidification of the cytoplasm stimulated the hydrolytic activity of adenosinetriphosphatase (ATP phosphohydrolase; EC 3.6.1.3) resulting in rapid hydrolysis of cytoplasmic ATP.

Several determinations failed to indicate any generalized loss of membrane integrity. In addition to the failure to detect release of ATP, there was no release of compounds absorbing at 260 or 280 nm, nor was there any increase in cell permeability to inulin or Na⁺ following treatment with nigericin. There was, however, a significant decrease in the activity of a cytoplasmic enzyme, β -galactosidase, after nigericin treatment. This decrease was slower than the effect of nigericin on viability, respiration or ATP, and control organisms exhibited a decrease in β -galactosidase activity that was almost as great (results not shown).

Since nigericin abolishes the transmembrane Δ pH by facilitating an electroneutral exchange of H⁺ and K⁺ (Asghar *et al.*, 1973; Harold & Baarda, 1968), the effects observed were possibly due to H⁺ influx and/or K⁺ efflux rather than solely to H⁺ influx. The effect of CCCP on the viability of *B. acidocaldarius* was therefore examined. This proton conductor abolishes the Δ pH in *B. acidocaldarius* (Krulwich *et al.*, 1978) and other organisms by

Fig. 2. Electron micrographs of organisms from control and nigericin-treated suspensions. The bacteria were incubated for 2 min at 50 °C in 25 mM-sodium citrate buffer, pH 3.5, in the absence (a) or presence (b) of 0.2 μ M-nigericin. Bar markers represent 0.5 μ m.

facilitating an electrogenic influx of protons which is not, at least initially, accompanied by a stoichiometric loss of K^+ . Under conditions in which 10 μ M-CCCP was used to treat the organisms instead of nigericin, a killing curve was generated that was identical to that found with nigericin (results not shown). It was still possible, however, that while proton influx was the critical factor in nigericin-induced death of *B. acidocaldarius*, the abolition of the Δ pH *per se*, rather than acidification of the cytoplasm, was the crucial event. Therefore, a series of incubations was conducted in buffers of increasing pH such that the Δ pH would be diminished and finally abolished without acidification of the cytoplasm. As shown in Table 1, incubation of organisms for 10 min at pH values as high as 6.0 (at which Δ pH would be zero) did not cause a loss of viability. The effect of nigericin diminished with increasing external pH. This decrease in effect was especially dramatic above pH 4.5, which is also close to the upper pH limit for growth and β -galactoside transport (Krulwich *et al.*, 1978). That this pH value is a transition point may relate to the pK of amino acid

Fig. 3

Fig. 4

Fig. 3. Effect of nigericin treatment on oxygen consumption by *B. acidocaldarius*. Organisms were incubated at 50 °C in 25 mM-sodium citrate buffer, pH 3.5, in the absence (○) or presence (●) of 0.2 μ M-nigericin. Samples were taken at the indicated times and assayed for oxygen consumption in the presence of 3 mM-lactose.

Fig. 4. Effect of nigericin on the cytoplasmic content of ATP. Organisms were suspended in 25 mM-sodium citrate buffer, pH 3.5, at 50 °C in the absence (○) or presence (●) of 0.2 μ M-nigericin. Samples were removed at the indicated times and assayed for cytoplasmic ATP content.

Table 1. *Effect of external pH on nigericin-induced death*

Organisms were suspended at equal concentrations in 25 mM-sodium citrate buffers at the indicated pH. Nigericin was added to a final concentration of 0.2 μ M to one suspension at each pH. All the suspensions were incubated for 10 min with shaking at 50 °C, then diluted (1 in 10⁴) in 25 mM-sodium citrate buffer, pH 3.5, and plated.

Buffer pH	10 ⁻⁶ × no. of colony-forming organisms after 10 min		Percentage of organisms killed
	Control	Nigericin-treated	
3.5	157	32	80
4.0	159	51	68
4.5	174	81	53
5.0	170	123	28
5.5	166	144	13
6.0	170	138	19

residues on both sides of the membrane which could affect membrane function. As shown in Fig. 5, the effect of nigericin also decreased as the incubation temperature was lowered. This was consistent with previous findings in this and other experimental systems (Krasne *et al.*, 1971; Krulwich *et al.*, 1978; Racker & Hinkle, 1974).

We thank Dr Saul Puszkin and his associates William Bloom and Christine Ores for their help with electron microscopy. This work was supported by research grant PCM 7810213 from the National Science Foundation. Dr Krulwich is the recipient of a Research Career Development Award from the National Institutes of Health. Dr Guffanti is a trainee on Institutional Training Grant GM 07036 from the National Institutes of Health.

Fig. 5. Effect of nigericin on the viability of *B. acidocaldarius* at various temperatures. Organisms were suspended at equal concentrations in sodium citrate buffer, pH 3.5, and incubated at the temperatures indicated for 10 min with 0.2 μ M-nigericin. Samples were then removed, plated and colony-forming units were counted.

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