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The vacuolar ATPase proton pump is required for the cytotoxicity of *Bacillus anthracis* lethal toxin

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Abstract The nature of the cytopathic effect exerted by the lethal factor toxin (LF) of *Bacillus anthracis* on sensitive cells is unknown. The toxin requires the passage through acidic vesicles in order to exert its effect within the cytosol. Here, we show that bafilomycins and concanamycin A, selective inhibitors of the vacuolar ATPase proton pump, are the most powerful known inhibitors of LF macrophage toxicity. These inhibitors are fully active long after LF addition to macrophages, suggesting that LF enters the cytosol after having reached a late endosomal compartment.

Key words: Lethal factor; Anthrax; Toxin; Translocation; V-ATPase; Endosome

1. Introduction

Bacillus anthracis, a Gram-positive bacterium, produces three protein toxin components, which are non-toxic if tested separately: protective antigen (PA, 83 kDa), edema factor (EF, 89 kDa) and lethal factor (LF, 83 kDa) [1]. PA is necessary for the activity of EF and LF to be displayed inside intact cells. EF is a calmodulin-dependent adenylate cyclase [2,3], whereas the biochemical mechanism of action of LF remains unknown.

Intoxication of macrophage cell lines by LF starts with PA binding to its cellular receptor. PA is then proteolytically cleaved by furin or furin-like cell proteases [4,5] and LF binds to the receptor bound C-terminal fragment of PA (63 kDa). The LF-PA63 complex is internalized by receptor-mediated endocytosis and appears to enter the lumen of acidic intracellular compartments, based on the observation that lysosomotropic agents prevent the cytotoxic activity of LF on macrophage cell lines [6-9]. Among the different cell types of an organism, LF is particularly active towards macrophages [10].

Bafilomycins (A1, B1, C and D) and concanamycin A are specific inhibitors of the vacuolar ATPase proton pump (V-ATPase) [11,12]. This pump is responsible for the lumenal acidic pH of several intracellular compartments including early and late endosomes and lysosomes [13,14]. These drugs inhibit proton flow through the proton channel of the V-ATPase [15] and have been increasingly used to probe the role of the V-ATPase in organelle acidification and of intracellular transmembrane pH gradients in several cell processes [13,14,16-19]. Here, we show that concanamycin A and bafilomycins are the most powerful inhibitors of LF cytotoxicity and that they are active long after the addition of LF to cells.

2. Materials and methods

2.1. Chemicals and reagents

Concanamycin A, bafilomycins A1, B1, C1 and D were prepared and purified as described previously [11,12]. They were dissolved in dimethyl sulfoxide at a concentration of 1 mM and stored in aliquots at −80°C. Their concentrations were determined as in [11] using the respective molar extinction coefficients published by Werner and Hagenmaier [20] for bafilomycin B1 and C1 and by Dröse et al. [12] for concanamycin A, bafilomycin A1 and D. [^{125}I]Na and 1,3,4,6-tetrachloro-3,6-diphenylglycouryl (IODO-GEN) were from Amersham International (Amersham, UK) and from Sigma (St. Louis, MO, USA), respectively.

2.2. Cell culture

The toxin-sensitive murine macrophage-like cell lines J774.A1 and RAW264.7 were obtained from the American Type Culture Collection (ATCC TIB 67, Rockville, MD, USA) and from the European Collection of Animal Cell Culture (ECAAC 91062702, Salisbury, UK), respectively. They are maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, penicillin (200 μg/ml) and streptomycin (100 μg/ml) and kept at 37°C in a 5% CO_2 atmosphere.

2.3. Toxin

PA and LF were obtained from the culture supernatant fluids of the *Bacillus anthracis* strains RP42 and RP4, respectively [21], and purified as described in [22]. Briefly, culture supernatants equilibrated in 20 mM Tris-HCl, pH 6.5 buffer were loaded onto the Mono-Q HR column (Pharmacia, Uppsala, Sweden). Proteins were eluted in the same buffer at 1 ml/min and 4°C with a 30 ml NaCl linear gradient (0–400 mM).

LF was radiolabelled with ^{125}I by the IODO-GEN method according to the procedure of described in [23]; specific activity of [^{125}I]LF was 10^7 cpm/μg. LF cytotoxicity was not affected by this iodination procedure.

2.4. Cell binding and internalization of [^{125}I]LF

Experiments were performed at 4°C in DMEM pH 7.2 without sodium bicarbonate and with 10% fetal calf serum: J774.A1 cells were incubated for 1 h without or with concanamycin A (5×10^{-8} M) or bafilomycin A1 (5×10^{-7} M) in the absence or presence of PA (500 ng/ml). Cells were then washed with medium and incubation was continued for 1 h with [^{125}I]LF (final concentration: 50 ng/ml) and inhibitors. Cells were washed again and incubated at 37°C with pre-warmed medium in the presence or in the absence of 3 mg/ml pronase (Boehringer, Mannheim, Germany). Such a treatment releases in the medium all cell surface-associated [^{125}I]LF. In the samples not containing pronase, it was added after 30 min and incubation was prolonged for an additional 30 min. Detached cells were recovered by centrifugation, and radioactivity in the pellet (internalized LF) and in the supernate (cell surface-associated LF) was counted.

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Abbreviations: PA, protective antigen; LF, lethal factor; EF, edema factor; PBS, phosphate buffered saline; DMF, dimethylformamide; SDS, sodium dodecyl sulfate; BafA1, bafilomycin A1; Conc A, concanamycin A; V-ATPase, vacuolar ATPase proton pump

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2.5. Cytotoxicity experiments

Cytotoxicity of LF was determined with the method described by Hansen et al. [24]. Briefly, cells were harvested by scraping with a rubber policeman and plated at a density of 5×10^4 cells/well in 96-well microtiter plates. When macrophages had reached confluence (after 2–3 days), PA (500 ng/ml) and various amounts of LF, LF plus inhibitor or inhibitor alone were added, and incubation was continued for 4 h. 30 ml of tetrazolium salt (4 mg/ml PBS) were then added and after 1.5 h incubation, the reaction was stopped by addition of 100 ml of stop buffer (20% w/v SDS, 50% v/v DMF, pH 4.7). Plates were shaken overnight at room temperature and absorbance was measured at 534 nm in a multiwell plate reader.

2.6. Neutral red uptake

The method previously described by Papini et al. was followed [18]. Briefly, cells were plated as described above in 96-well microtiter plates. Experiments were carried out with or without the various drugs, and then the Neutral red solution (0.05% w/v Neutral red, 0.3% w/v BSA in PBS) was added to the cells for 5 min at 37°C. Cells were then washed three times with PBS, 3% w/v BSA. Neutral red was extracted using acidified ethanol (70% ethanol, 0.37% HCl). Readings were taken at 534 nm with a microwell plate reader.

3. Results and discussion

Previous experiments have shown that LF has to enter an intracellular acidic compartment in order to display its toxic activity in the cytosol of macrophages [6-9]. This conclusion was derived from the protective effect exerted by lysosomotropic agents such as ammonium chloride, monensin and chloroquine. The action of these agents is not restricted to endosomes and lysosomes, and therefore some of their cellular effects may be not directly correlated with neutralization of intracellular acidic compartments. On the contrary, the macrolide antibiotics concanamycin A (conc A) and bafilomycins A1 (bafA1), B1, C1 and D were characterized as specific and powerful inhibitors of the V-ATPase which is responsible for acidification of the lumen of endosomal and lysosomal compartments. Fig. 1 shows that these drugs added to the macrophage cell line J774A.1 inhibit the cytotoxic activity of LF. Overlapping results were obtained with the RAW264.7 macrophage cell line (Table 1). Their order of potency of the different bafilomycins correlates with their ability to inhibit the V-ATPase [12]. Fig. 2 shows that bafA1 and concanamycin do not affect either fixation or cell entry of [^{125}I]LF into J774A.1 cells. These experiments identify conc A and bafA1 as the two most powerful inhibitors of LF macrophage cytotoxicity. Their efficacy and lack of known effects on other cellular targets, in the range on concentrations used here, suggest that they will be very useful in studies on the molecular mechanism of cell intoxication by LF.

These results also indicate that LF enters into macrophages

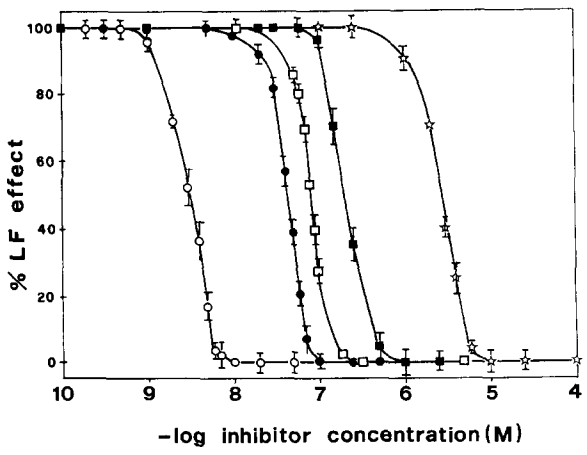

Fig. 1. Inhibition of the cytopathic effect of LF. J774.A1 cells were incubated with the indicated concentrations of concanamycin A (○), bafilomycins A1 (●), B1 (□), C1 (■) and D (☆) for 1 h and then treated with PA (500 ng/ml) and LF (100 ng/ml) for an additional 4 h. Cell death was determined as described in Section 2 and expressed as percentage of the LF effect induced in cells not treated with inhibitor. Values are the mean of three experiments run in triplicates and bars represent ± SE.

Table 1
Comparison of bafilomycins and concanamycin A inhibition of anthrax lethal factor cytotoxicity on J774.A1 and RAW264.7 macrophage cell line and of *Neurospora crassa* V-ATPase

Inhibitor	IC_{50} (M)		*IC_{50} (μmol/mg)
	J774.A1	RAW264.7	N.c. V-ATPase[a]
Concanamycin A	2.8×10^{-9}	3.4×10^{-9}	2×10^{-6}
Bafilomycin A1	4.4×10^{-8}	3.5×10^{-8}	5×10^{-5}
Bafilomycin B1	8.2×10^{-8}	6.6×10^{-8}	n.d.
Bafilomycin C	2.1×10^{-7}	1.8×10^{-7}	n.d.
Bafilomycin D	2.7×10^{-6}	n.d.	2×10^{-3}

[a]IC_{50} values were calculated with the fit function of the 'origin' program and did not depend on the LF concentration in the 50–500 ng/ml range. Values for *N. crassa* V-ATPase are taken from [13].

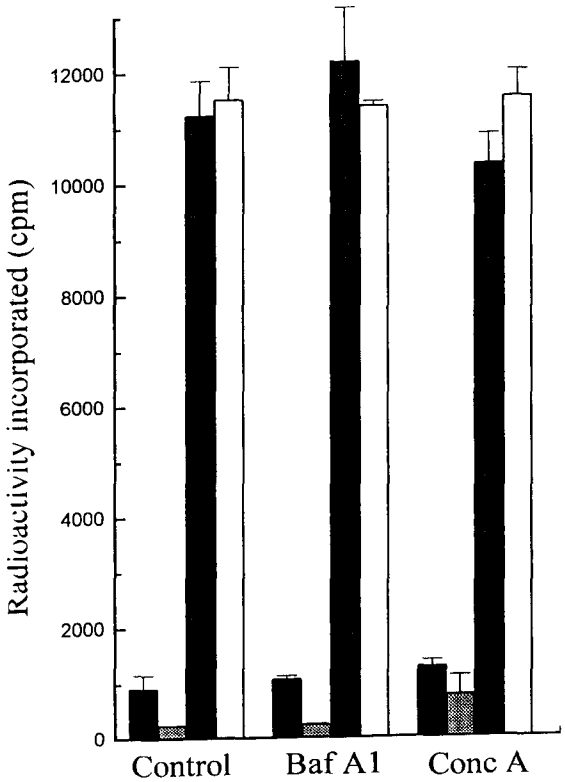

Fig. 2. Effect of bafilomycin A1 and concanamycin A on ^{125}I-LF binding of to macrophage cells. J774.A1 cells were incubated at 4°C without or with concanamycin A (5×10^{-8} M) or bafilomycin A1 (5×10^{-7} M) in the absence or presence of PA (500 ng/ml) followed by a 1 h incubation with [^{125}I]LF (50 ng/ml). Bound [^{125}I]LF was either stripped from the cell surface with pronase, or internalized by incubation at 37°C. Black and dark gray bars represent cell bound [^{125}I]LF in the absence and presence of PA, respectively. Light gray and empty bars represent cell internalized [^{125}I]LF in the absence and presence of PA, respectively. Data are average of three independent experiments and bars give SD values.

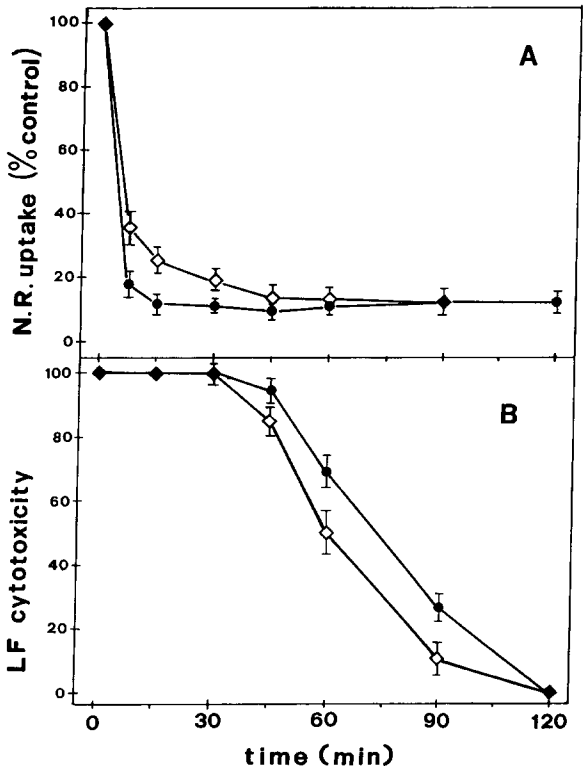

Fig. 3. Effect of bafilomycin A1 and bafilomycin A1 plus monensin on neutral red uptake (A) and LF cytotoxycity (B) of macrophage cells. (A) J774.A1 cells were incubated for the time periods indicated in abscissa with bafilomycin A1 (5×10^{-7} M) (◇) or bafilomycin A1 (5×10^{-7} M) plus monensin (10^{-5} M) (●) and then neutral red uptake was measured, as described in Section 2. Data are expressed as percentage of the dye uptake in untreated cells. Values are the mean of five experiments run in triplicate and bars represent SE. (B) J774.A1 cells were incubated with LF and PA (500 ng/ml each) at 37°C at time 0. After the time periods indicated on the abscissa, bafilomycin A1 (5×10^{-7} M) (◇) or bafilomycin A1 (5×10^{-7} M) plus monensin (10^{-5} M) (●) were added and incubations were prolonged for a further 4 h. Cell death was determined and expressed as percentage of the cytopathic effect caused by LF in cells not treated with inhibitor. Values are the mean of three experiments run in triplicate and bars represent SE.

following a pathway of receptor-mediated endocytosis into compartments endowed with a V-ATPase proton pump. Clathrin-coated vesicles, early and late endosomes and lysosomes contain the V-ATPase [25]. Panels A of Figs. 3 and 4 show that conc A and bafA1 prevent the acidification of intracellular acidic compartments that become unable to take up the membrane permeant weak base Neutral red. The kinetic analysis of Fig. 3A shows that, within 15 min in the presence of bafA1, J774.A1 cells no longer possess transmembrane pH gradients. Monensin quenches transmembrane pH gradients by altering the membrane permeability barrier to protons [26] and is used here to accelerate the action of bafilomycin A1. In the presence of both monensin and bafilomycin A1, the time required to quench transmembrane pH gradients is reduced to less than 10 min. Such a rapid action of these drugs allows one to obtain information on when LF enters the acid intracellular compartment, after its cell surface binding. In fact, these drugs will prevent LF cytotoxicity as long as LF has not entered the intracellular acidic compartment where it undergoes the low pH-driven structural change that enables it to translocate into the cytosol, where its exerts its toxic activity [22]. Panels B of Figs. 3 and 4 show that drugs that prevent acidification of intracellular acidic compartments also prevent LF intoxication of J774.A1 cells long after LF addition to cells. Under the conditions of most rapid quenching of intracellular acidic pH, i.e. bafilomycin A1 plus monensin, cells are protected for more than 1 h. Cell ligands such as EGF or LDL enter early endosomal compartments within few minutes and move to late endosomal compartments within 20–30 min [27,28]. Based on these data, the present results indicate that LF undergoes the low pH-dependent step of its cell intoxication process in late endosomal compartments. This work does not shed light on the intracellular compartment from which LF escapes into the cytosol. But it indicates that the acidic organelle where LF becomes competent to cross the membrane is a late endosomal compartment. The actual translocation of LF into the cytosol could take place in late endosomes themselves as well as in the trans Golgi compartment which is dynamically linked to late endosomes via a membrane trafficking pathway controlled by rab9 [29,30]. This is at variance from what was found before with diphtheria toxin, which is the best characterized protein toxin requiring a transmembrane pH gradient to translocate its catalytic subunit into the cell cytosol. Membrane translocation of

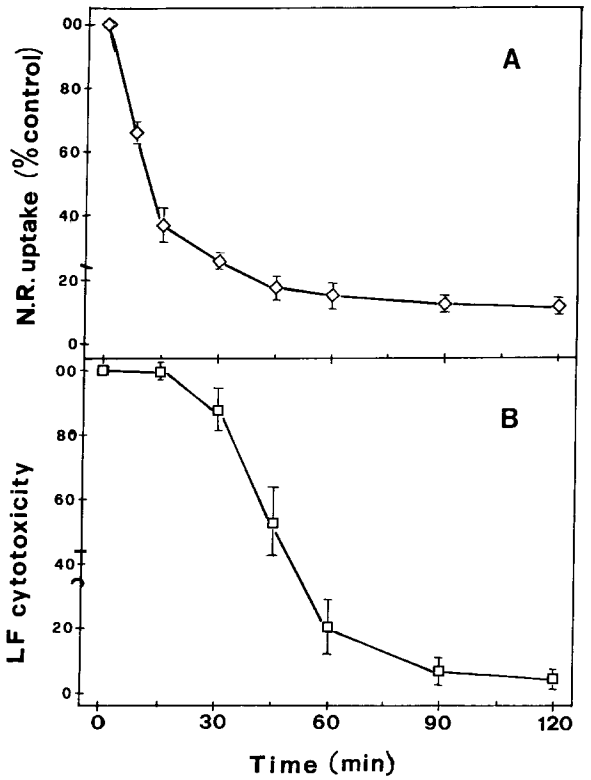

Fig. 4. Effect of concanamycin A on the cell accumulation of Neutral red (A) and on prevention of LF cytotoxycity (B). (A) J774.A1 cells were incubated for the time periods indicated on the abscissa with concanamycin A (5×10^{-8} M) and Neutral red uptake was measured as described in Section 2. Data are expressed as percentage of the dye uptake in untreated cells. Values are the mean of five experiments run in triplicate and bars represent SE. (B) J774.A1 cells were incubated with LF and PA (500 ng/ml each) at 37°C at time 0. After the time periods indicated on the abscissa, concanamycin A (5×10^{-8} M) was added and incubations were prolonged for a further 4 h. Cell death was determined and expressed as percentage of the cytopathic effect caused by LF in cells not treated with inhibitor. Values are the mean of three experiments run in triplicate and bars represent SE.

diphtheria toxin takes place within 10 min after the onset of endocytosis and is prevented by bafA1 only for few minutes after toxin internalization [16,17]. Confocal and electron microscopy studies are required to investigate the exact pathway followed by LF to entry into cells. Present results indicate that LF shares with diphtheria toxin the initial steps of binding and endocytosis into early endosomes, but differ afterwards.

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