INFECTION AND IMMUNITY, June 1997, p. 2240–2242 Vol. 65, No. 6
0019-9567/97/$04.00+0

Elevation of Vacuolar pH Inhibits the Cytotoxic Activity of Furin-Cleaved Exotoxin A

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Received 22 November 1996/Returned for modification 28 February 1997/Accepted 25 March 1997

Exotoxin A (ETA) inhibits protein synthesis in cells by a process that involves receptor-mediated endocytosis and the transport of a 37-kDa proteolytic fragment across a membrane into the cytoplasm. The fragment is apparently generated by the endoprotease furin after the toxin has been endocytosed. Cleavage of ETA by furin requires a low pH in vitro, and presumably also in vivo. Drugs that raise the pH of intracellular compartments are known to protect cells from ETA. The simplest hypothesis to explain this protection has been that the drugs interfere with furin cleavage. To test this idea, we measured the effect of pH-elevating drugs on the action of ETA that had been precleaved with recombinant furin before addition to cells. Surprisingly, we found that pH-elevating drugs protected cells from precleaved ETA as well as intact ETA. These results suggest that the process by which ETA intoxicates cells requires a low vacuolar pH for another event in addition to proteolysis by furin.

Pseudomonas aeruginosa exotoxin A (ETA) (molecular weight = 66,583) catalyzes the transfer of the ADP-ribosyl moiety of NAD^+ to elongation factor 2 in mammalian cells, which inactivates elongation factor 2 and arrests protein synthesis, killing the cell (25). To access elongation factor 2, ETA must be transported across a membrane and into the cytoplasm. The first step in the transport process is receptor-mediated endocytosis, which brings the toxin into the vacuolar compartment of the cell. The cell surface receptor for ETA is the α_2-macroglobulin receptor/low density lipoprotein receptor-related protein (8). Events subsequent to endocytosis that result in delivery of the toxin to the cytoplasm are not well understood; however, it is known that ETA is cleaved by the protease furin between residues Arg279 and Gly280 to produce an N-terminal fragment of about 29 kDa and a C-terminal fragment of about 37 kDa (6, 14, 19, 20). The C-terminal fragment contains the catalytic center and is the minimal portion of the toxin that must reach the cytoplasm to inhibit protein synthesis.

Drugs that elevate the pH within vacuolar compartments, such as lysosomotropic amines, inhibit the cytotoxic activity of ETA (13). Moreover, mutations that impair vacuolar acidification in mammalian cells also block cytotoxic activity (11, 12, 15). It is inferred from these observations that some critical event in the entry process requires a low pH. While furin has a broad pH optimum with model peptides as substrates (16), the optimum with ETA as a substrate is between pH 5.0 and 5.5 (3), apparently due to a conformational change in the toxin at low pH that exposes the cleavage site. Therefore, one explanation for the low pH requirement during the intoxication process in vivo has been that it is necessary for the proteolytic cleavage of ETA. To test this idea, we measured the effect of pH-elevating drugs on the cytotoxic activity of ETA that had been precleaved with furin before addition to cells, with the expectation that the drugs would not inhibit precleaved ETA. Surprisingly, the cytotoxic activity of precleaved toxin was still strongly inhibited by agents that elevate the pH in vesicles, suggesting that an event in addition to furin cleavage is dependent on a low pH.

(Work by M. J. Corboy partially fulfills the requirements for the Ph.D. degree in Molecular and Cell Biology.)

MATERIALS AND METHODS

Materials. ETA was a gift from Stephen Leppla (National Institute of Dental Research, National Institutes of Health, Bethesda, Md.). A soluble form of recombinant human furin, lacking the C-terminal 81 amino acids (16), was a gift from Gary Thomas (Vollum Institute, The Oregon Health Science University, Portland, Oreg.). Monensin, bafilomycin A1, and brefeldin A were purchased from Sigma Chemical Corp. (St. Louis, Mo.) and stored as concentrated stocks in dimethyl sulfoxide. Rabbit anti-ETA immunoglobulin G (IgG) was prepared by 70% ammonium sulfate precipitation from rabbit anti-ETA antiserum as described by Mozola et al. (18).

Cells. Thymidine kinase-deficient mouse L cells ($LMTK^-$) were grown at 37°C in humidified 10% CO_2–90% air in Dulbecco's modified Eagle's medium supplemented with 5% FetalClone II (Hyclone) and 10 mM HEPES, pH 7.4. For protein synthesis inhibition assays, cells were plated in 48-well plates 1 to 3 days in advance to give about 10^5 cells per well at the time of the assay.

In vitro cleavage of ETA with furin. ETA was treated with furin in reaction mixtures containing 3 mM $CaCl_2$, 0.1% bovine serum albumin, 1.0 μM NAD^+, and either 50 mM sodium acetate, pH 5.4, or 10 mM HEPES, pH 7.4. The reaction mixtures were incubated overnight at room temperature. Cleavage was assessed by immunoblotting with anti-ETA antibodies. ETA (5 ng) was electrophoresed in 10% polyacrylamide gels with sodium dodecyl sulfate and transferred to Immobilon-NC membranes (Millipore) by using a Transphor Electrophoresis Unit (Hoefer). The membranes were blocked with 1% nonfat dry milk in Tris-buffered saline, probed with the anti-ETA IgG, developed with the Amersham horseradish peroxidase-protein A enhanced chemiluminescence Western blotting analysis system, and exposed to HyperFilm.

Protein synthesis inhibition assays. The cytotoxicity of ETA was assayed essentially as described by Chaudry et al. (2). The growth medium on $LMTK^-$ cells was replaced with 0.25 ml of methionine-deficient Dulbecco's modified Eagle's medium lacking bicarbonate, and various amounts of either untreated ETA or furin-cleaved ETA were added in the presence or absence of the indicated concentrations of drugs. The cells were incubated for 4 h at 37°C in air, with the last hour including 0.5 μCi of [^{35}S]methionine (Tran^{35}S-Label; ICN)/well. Protein synthesis was measured as the incorporation of label into acid-precipitable material and quantitated on a PhosphorImager (Molecular Dynamics) with ImageQuant analysis software.

RESULTS AND DISCUSSION

The in vitro cleavage of ETA by furin is shown in Fig. 1. Samples of ETA were incubated without furin, with furin at pH 7.4, or with furin at pH 5.4. The toxin was electrophoresed in

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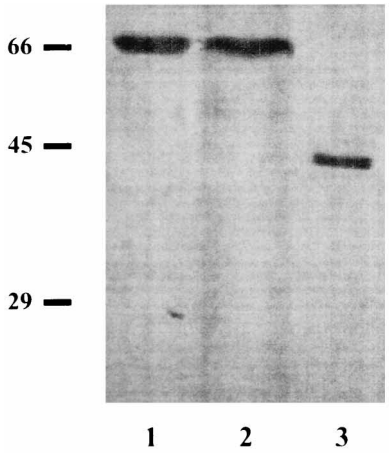

FIG. 1. Proteolysis of ETA by furin. ETA (230 μg/ml) was incubated with furin (35 μg/ml) overnight at room temperature in either acidic or neutral buffer. Reaction products were analyzed by electrophoresis in polyacrylamide gels with sodium dodecyl sulfate and mercaptoethanol followed by immunoblotting with anti-ETA IgG as described in Materials and Methods. Lane 1, untreated ETA; lane 2, ETA treated with furin, pH 7.4; lane 3, ETA treated with furin, pH 5.4. The sizes of molecular mass markers are indicated in kilodaltons.

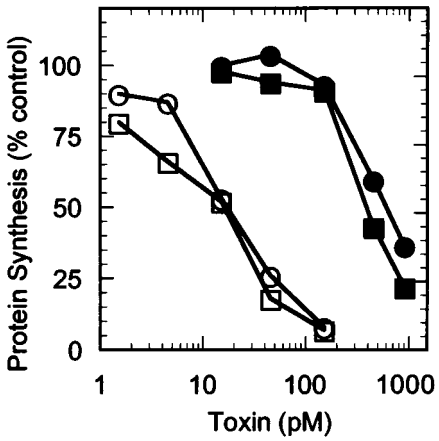

FIG. 2. Effect of ammonium chloride on cytotoxic activity of intact and furin-cleaved ETA. LMTK$^-$ cells were incubated for 4 h with various concentrations of intact ETA (circles) or furin-cleaved ETA (squares) in the presence (filled symbols) or absence (open symbols) of 20 mM ammonium chloride. Protein synthesis was measured by the incorporation of [^{35}S]methionine into acid-precipitable material during the final hour of incubation and is expressed as a percentage relative to control cells that received no toxin. Each data point represents the average of triplicate values from three separate experiments.

a polyacrylamide gel with sodium dodecyl sulfate and mercaptoethanol, transferred to nitrocellulose, and detected by immunoblotting. Incubation with furin at pH 7.4 (lane 2) resulted in no visible cleavage of ETA, emphasizing the importance of a low pH for furin activity on ETA. Incubating ETA with furin at pH 5.4 (lane 3) gave quantitative cleavage to produce the 37-kDa C-terminal fragment visible in lane 3. The 29-kDa N-terminal fragment is not visible in lane 3 because the antibody used in the immunoblots reacts with epitopes present predominantly in the C-terminal end of ETA. Furin-cleaved ETA for subsequent experiments was prepared by incubation with furin at pH 5.4 as in lane 3 of Fig. 1.

To compare the effects of ammonium chloride on the ability of furin-cleaved and uncleaved ETA to inhibit protein synthesis, mouse LMTK$^-$ cells were exposed to the toxins in the presence and absence of ammonium chloride for 4 h. Protein synthesis was assessed by the incorporation of radioactive methionine into acid-insoluble protein during the last hour of the incubation. The 50% inhibitory concentration (IC_{50}) for both cleaved and uncleaved ETA in the absence of ammonium chloride was 15 to 17 pM (Fig. 2 and Table 1), demonstrating that furin cleavage of ETA had no adverse effect on the ability of the toxin to bind to receptors and enter the cytoplasm. In the presence of ammonium chloride, the IC_{50} for uncleaved ETA was 631 pM and that for furin-cleaved ETA was 390 pM. Thus, ammonium chloride inhibited ETA regardless of whether the toxin was intact or cleaved with furin before addition to the cells.

Ammonium chloride is a lysosomotropic amine and raises the pH inside acidic vesicles by acting as a proton trap (4). Methylamine and chloroquine, which are also lysosomotropic amines, are reported to inhibit the cytotoxic activity of ETA by blocking endocytic uptake rather than by blocking a later step dependent on a low pH within an intracellular compartment (5, 17). To corroborate that the effect of ammonium chloride on ETA was related to a change in vesicular pH, we measured the effect on ETA action of other drugs that elevate intracellular pH by mechanisms different from that of lysosomotropic amines. Monensin is a carboxylic ionophore and elevates the pH within acidic compartments by exchanging protons for sodium ions across the membrane (24). Bafilomycin A1 raises the pH by directly inhibiting the proton-translocating ATPase that pumps protons into the vesicles (1). As seen in Table 1, both monensin and bafilomycin A1 inhibited the effect of ETA on protein synthesis regardless of whether the toxin was intact or cleaved by furin. It is likely, therefore, that inhibition of ETA action by lysosomotropic amines, monensin, and bafilomycin A1 is related to their common effect on intravesicular pH.

We also compared the effects of brefeldin A on the cytotoxic activity of furin-cleaved and intact ETA. Brefeldin A blocks the binding of coat proteins to Golgi membranes, which disrupts membrane traffic through the Golgi complex (9, 10, 21). Brefeldin A also inhibits the entry of ETA into the cytoplasm (7, 26), which is part of the evidence supporting the proposal that ETA passes through the Golgi complex by retrograde transport en route to the cytoplasm (22, 23). However, it is also possible that brefeldin A disrupts membrane traffic such that ETA and furin do not meet in the presence of brefeldin A. If this were the basis for brefeldin A-mediated inhibition of ETA action, then furin-cleaved ETA might not be inhibited by

TABLE 1. Effects of ammonium chloride, monensin, bafilomycin A1, and brefeldin A on cytotoxic activity of ETA

ETA	Drug (concn)	IC_{50} (pM)[a]
Intact	None	17.4 ± 0.9
	Ammonium chloride (20 mM)	631 ± 42
	Monensin (20 μM)	>900
	Bafilomycin A1 (0.5 μM)	>900
	Brefeldin A (5 μM)	>900
Furin-cleaved	None	15.0 ± 0.8
	Ammonium chloride (20 mM)	390 ± 23
	Monensin (20 μM)	>900
	Bafilomycin A1 (0.5 μM)	>900
	Brefeldin A (5 μM)	>900

[a] The IC_{50} is the concentration of ETA required to reduce protein synthesis by 50%. Values are means of triplicate samples from at least two separate experiments ± the standard errors of the mean. For IC_{50}s greater than the highest concentration of toxin used (900 pM) the value is given as >900.

brefeldin A. The results in Table 1 show, however, that brefeldin A still inhibits furin-cleaved ETA. Thus, the inhibitory effect of brefeldin A on the access of ETA to the cytoplasm cannot be attributed solely to a failure of furin to cleave ETA in the presence of brefeldin A.

In summary, the main observation reported here is that the transport of furin-cleaved ETA to the cytoplasm is still inhibited by elevating the pH inside intracellular vesicles. This suggests that more than one event in the entry process depends on a low vacuolar pH. Since the pH optimum for proteolysis of ETA by furin is acidic, certainly one step that should be sensitive to raising the pH in vivo is cleavage of ETA by furin. By giving cells precleaved ETA, we were able apparently to uncover a second event dependent on a low pH. The nature of the second event is not clear. One possibility is that dissociation of ETA from its receptor after endocytosis is necessary before the toxin can proceed to the cytoplasm, and this dissociation does not occur unless the pH is low. A second possibility is that the acid-induced conformational change in ETA that is necessary for furin cleavage is necessary in addition for ETA to pass through a membrane; even precleaved ETA might not penetrate a membrane unless the pH is low. In this regard, however, we have been unable to enhance the entry of furin-cleaved toxin into the cytoplasm by acidifying the medium bathing cells to which furin-cleaved ETA is bound. Further work is needed to identify and understand the second pH-sensitive event in the mechanism of ETA action.

ACKNOWLEDGMENTS

This research was supported in part by Public Health Service grant GM-34297 from the National Institute of General Medical Sciences.

We are indebted to G. Thomas for providing recombinant furin.

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Editor: J. R. McGhee