***Bordetella pertussis* adenylate cyclase inactivation by the host cell**

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Bordetella pertussis produces a calmodulin-dependent adenylate cyclase (AC) which acts as a toxin capable of penetrating eukaryotic cells and generating high levels of intracellular cyclic AMP. Transfer of target cells into *B. pertussis* AC-free medium leads to a rapid decay in the intracellular AC activity, implying that the invasive enzyme is unstable in the host cytoplasm. We report here that treatment of human lymphocytes with a glycolysis inhibitor and an uncoupler of oxidative phosphorylation completely blocked the intracellular inactivation of *B. pertussis* AC. Lymphocyte lysates inactivated all forms of *B. pertussis* AC in the presence of exogenous ATP. This inactivation was associated with degradation of an ¹²⁵I-labelled 200 kDa form of *B. pertussis* AC. It appears that ATP is required for the proteolytic pathway, but not as an energy source, since non-hydrolysable ATP analogues supported inactivation and complete degradation of the enzyme. The possibility that binding of ATP to *B. pertussis* AC renders it susceptible to degradation by the host cell protease is discussed.

INTRODUCTION

Bordetella pertussis, the causative agent of whooping cough, produces adenylate cyclase (AC) whose properties differ from those of other prokaryotic enzymes in being largely extracytoplasmic and stimulated by the eukaryotic Ca²⁺-binding protein, calmodulin (CaM) [1,2]. It is well documented that *B. pertussis* AC is capable of penetrating mammalian cells and generating high levels of cAMP within them [3–5]. In immune effector cells, the unregulated generation of cyclic AMP impairs functions such as chemotaxis, superoxide generation and microbial killing [6]. It was therefore suggested that *B. pertussis* AC acts as a toxin which suppresses host defences and assists the survival of the bacterium [6]. The importance of AC as a virulence factor was directly demonstrated by the findings that a transposon insertion mutant of *B. pertussis* deficient in AC was avirulent [7].

Recently, several laboratories have described significant purification of *B. pertussis* AC. Ladant *et al.* [8] have purified the enzyme from bacterial culture medium; three structurally related catalysts [9] of molecular masses 43, 45 and 50 kDa were isolated. Rogel *et al.* [10] purified from extracts of concentrated bacterial cells two catalysts, of molecular masses 200 and 47 kDa, possessing immunologically related domains. Glaser *et al.* [11] have cloned and sequenced the *B. pertussis* gene for AC and showed that it has the potential to encode a very large polypeptide. Thus, the enzyme appears to be synthesized as a precursor of 200 kDa which is processed in the bacterium and secreted as lower molecular mass forms. The structure of the invasive form of the enzyme and whether it is delivered to target cells via direct contact with the bacterium or only after being released into the culture medium are not yet clear. The invasive form of the enzyme extracted from the bacteria migrated

on gel filtration as a distinct minor peak of AC activity with apparent size of 190 kDa [4]. Immunoblot analysis of this form revealed the presence of a major catalyst of 200 kDa and in some preparations additional breakdown products of lower molecular mass [10]. Masure *et al.* [12] resolved from bacterial culture medium both invasive and non-invasive forms of *B. pertussis* AC. Only the invasive form displayed a Ca²⁺-dependent increase in its size. The catalysts purified from these two forms both had molecular masses of 45 kDa, but were incapable of penetrating cells. Further studies are required to establish whether or not an additional component is required for the entry of the catalyst into cells.

The accumulation of the invasive form within mammalian cells proceeded without any noticeable lag time and reached a constant level with 10–30 min of cell exposure [5,13]. The constant level was maintained provided that the invasive enzyme was present in the medium. Upon transfer of cells into enzyme-free medium, a rapid decrease in the intracellular AC activity was observed [5,13]. This decrease reflected intracellular inactivation of the invasive enzyme and did not result from its release back to the medium. Since entry of polypeptides as well as their processing within eukaryotic cells have been reported to be energy-dependent events [14,15], we tested the effect of metabolic inhibitors on the penetration and on the intracellular inactivation of *B. pertussis* AC. Here we demonstrate that the decay of *B. pertussis* AC activity within human lymphocytes is completely prevented by depletion of cellular ATP. Broken-cell preparations proteolytically inactivate *B. pertussis* AC in the presence of millimolar concentration of exogenous ATP. The inactivation of *B. pertussis* AC by target cell proteases may represent a novel mechanism by which host cells protect themselves against intruding proteins.

Abbreviations used: AC, adenylate cyclase; CaM, calmodulin; PMSF, phenylmethanesulphonyl fluoride; TPCK, L-1-tosylamido-2-phenylethylchloromethyl ketone; App[NH]p, adenosine 5'-[β -imidotriphosphate; Gpp[NH]p, guanosine 5'-[β -imidotriphosphate; GTP[S], guanosine 5'-[γ -thiotriphosphate; PBS, phosphate-buffered saline; NP-40, Nonidet P-40; PAGE, polyacrylamide-gel electrophoresis.

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EXPERIMENTAL

Culture of organism and purification of *B. pertussis* AC

B. pertussis strain 165 was grown in Stainer-Scholte liquid medium at 37 °C as described previously [4]. The 47 kDa catalyst was purified according to the procedure of Rogel *et al.* [10]. The partially purified invasive form of AC was obtained by chromatography of crude dialysed urea extract of the bacteria on an Ultrogel AcA34 column as previously described [4,10]. A 200 kDa catalyst was purified from this form essentially according to the procedure described [10]. The enzyme was loaded on CaM-agarose column in a solution containing 50 mM-Tris, pH 7.4/100 mM-NaCl/0.2 mM-CaCl₂/2 M-urea. Then the column was washed with the following solutions: (1) 50 mM-Tris, pH 7.4/0.5 M-NaCl/0.2 mM-CaCl₂, (2) 50 mM-Tris, pH 7.4/2 mM-EGTA, (3) 50 mM-Tris, pH 7.4/0.2 mM-CaCl₂. The elution was performed with a solution containing 50 mM-Tris, pH 7.4/0.2 mM-CaCl₂/8.8 M-urea. The hydroxylapatite chromatography was omitted and the eluent was dialysed against a buffer containing 50 mM-Tris, pH 7.4/0.2 mM-CaCl₂ and concentrated by Amicon ultrafiltration using a PM-10 membrane. *Bacillus anthracis* Sterne strain was obtained from Dr. R. Tamarin, The Veterinary Institute, Beit Dagan, Israel. The Sterne strain was grown for 30 h at 36 °C in defined medium as described by Ristroph & Ivins [16]. The culture supernatant was separated from the bacteria and used as a crude preparation of *B. anthracis* AC.

Human lymphocytes and lymphocyte lysates

Human lymphocytes were isolated from whole blood as described previously [4]. Lysates from untreated cells or cells pre-exposed to the invasive enzyme were prepared as follows. Cells [(1–20) × 10⁷] were suspended in 5 ml of lysing solution containing 20 mM-Hepes, pH 7.5/2 mM-MgCl₂/2 mM-EDTA/2 mM-leupeptin/0.15 mM-pepstatin. The cells were then disrupted by freezing and thawing twice using liquid N₂ followed by homogenization with a Dounce homogenizer using tightly fitted pestles. Nuclei and unbroken cells were removed by centrifugation at 900 g (IEC centrifuge) for 10 min. The lysate contained 1–2 mg of protein/ml and was stable for 14 days when kept frozen at –135 °C.

Determination of the invasive-enzyme activity

Lymphocytes were incubated with the invasive form of *B. pertussis* AC and the activity of the enzyme within the cells was determined after treatment of cells with trypsin as we have previously described [4,5]. The intracellular decay in the activity of the invasive enzyme was monitored as explained previously [5,13]. The results shown represent a mean of three determinations and the coefficient of variation did not exceed 8%.

Inactivation of *B. pertussis* AC by lymphocyte lysates

The inactivation reaction was performed at 36 °C in a final volume of 50 µl and included (when performed in the presence of ATP) the following ingredients: 0.2–0.4 mg of cell lysate·ml⁻¹, 0.1% (w/v) Nonidet P-40 (NP-40)/1 µM-CaM/0.1 mM-CaCl₂/2 mM-MgCl₂/2 mM-EDTA/3 mM-ATP. In the absence of ATP, an ATP trap consisting of 10 mM-glucose and 1 unit of hexokinase was added. When lysates of non-treated cells were used, *B. pertussis* AC (various forms of activity of 1–

4 nmol·min⁻¹) or ¹²⁵I-labelled 200 kDa catalyst were added to the inactivation reaction. At various times, samples of 5–10 µl were withdrawn, diluted 10-fold into a solution containing 20 mM-Hepes, pH 7.5/2 mM-MgCl₂/1 mM-EDTA, and assayed in triplicate for AC activity. The results shown represent a mean of three determinations and the coefficient of variation did not exceed 6%. For visualization of the degradation of ¹²⁵I-labelled 200 kDa catalyst, 15 µl samples (containing about 50000 c.p.m. of ¹²⁵I-labelled protein) were withdrawn and mixed with 15 µl of concentrated sample buffer [17]. The samples were boiled for 3 min and then subjected to polyacrylamide-gel electrophoresis (PAGE) in SDS followed by autoradiography as explained below.

Iodination and PAGE

Iodination of *B. pertussis* AC was conducted essentially according to the procedure of Ladant [9]. The 200 kDa catalyst was purified as explained above, and prior to its elution from the CaM-agarose column, 70 µl of packed resin was withdrawn and used for iodination. The iodinated catalyst was eluted and then passed through 10 ml of a Sephadex G-25 column equilibrated and run in a solution containing 50 mM-Tris, pH 7.5/0.1 mM-CaCl₂/0.1% (w/v) NP-40 and 0.1 mg of bovine serum albumin·ml⁻¹. The activity of the iodinated catalyst was 0.1–0.2 µmol·min⁻¹·10⁻⁷ c.p.m. of ¹²⁵I.

PAGE was performed as described by Laemmli [17]. For visualization of the degradation products of ¹²⁵I-labelled AC, the gels were fixed, dried and autoradiographed at –70 °C for 1–12 h. For detection of AC activity in the gel, gel lanes were cut into 0.4 cm slices and the slices were treated with 2% CHAPS solution as described by Masure *et al.* [12]. After renaturation, AC activity and radioactivity were determined.

Other procedures

AC activity of *B. pertussis* and of *B. anthracis* was determined in a total volume of 50 µl, and the assay mixture contained 50 mM-Hepes, pH 7.6/1 mM-[α-³²P]-ATP/10 mM-MgCl₂/60 µM-CaCl₂/10 µM-CaM. Crude preparations of plasma membranes from rat brain and human lymphocytes were prepared according to the procedures of Minocherhomjee *et al.* [18] and Ross *et al.* [19] respectively. The AC enzymes were preactivated by incubation of the membranes at 30 °C for 20 min with 5 mM-MgCl₂/10 µM-guanosine 5'-[γ-thio]triphosphate (GTP[S]) and then extracted with 0.5% (w/v) Lubrol-PX as described [18]. The activities of the solubilized enzymes were determined in an assay mixture supplemented with 10 µM-forskolin, 10 µM-CaM and 10 µM-GTP[S] [19]. The results shown represent means of three determinations and the coefficient of variation did not exceed 10%. The [³²P]cAMP formed was isolated according to Salomon *et al.* [20]. Proteins were determined by the methods of Bradford [21] and Schaffner & Weissmann [22]. The levels of ATP in human lymphocytes before and after depletion were estimated by firefly luciferase assay [23].

Materials

AMP, ADP, ATP, GTP, CTP, cAMP, adenosine 5-[βγ-imido]triphosphate (App[NH]p), guanosine 5'-[βγ-imido]triphosphate, αβ-methylene-ATP, βγ-methylene-ATP, GTP[S], *N*-ethylmaleimide, *L*-1-tosylamido-2-phenylethylchloromethyl ketone (TPCK) and phenyl-

Fig. 1. Inactivation of *B. pertussis* AC in intact cells and cell lysates requires ATP

(a) Human lymphocytes (2×10^7) were incubated for 25 min at 36 °C in 8 ml of phosphate-buffered saline (PBS; 5 mM-sodium phosphate/140 mM-NaCl, pH 7.4) with 500 μ g of the partially purified invasive form of *B. pertussis* AC. Then the cells were divided to two equal pools. One pool (Δ) received 10 mM- NaN_3 and 50 mM-2-deoxyglucose and both pools were further incubated for 10 min. Then, the cells (of the two pools) were washed extensively and reincubated in toxin-free PBS in the presence (Δ) or the absence ($\circ$) of metabolic inhibitors. At the indicated times, 0.5 ml samples were withdrawn, transferred to ice and the intracellular AC activity was determined as described in the Experimental section. (b) Human lymphocytes (3×10^7) were incubated in 5 ml of PBS with 500 μ g

methanesulphonyl fluoride (PMSF), EDTA, leupeptin, pepstatin, bovine brain CaM, CaM-agarose, protein standards and hexokinase were obtained from Sigma. Ficoll-Hypaque was obtained from Pharmacia LKB Biotechnology Inc., Ultrogel AcA 34 from LKB and urea (fluorimetrically pure) was a product of Schwarz/Mann. [α - ^{32}P]ATP (80 Ci/mmol), [^3H]cAMP (27 Ci/mmol) and Na^{125}I (1000 Ci/mmol) were obtained from Amersham.

RESULTS

Inactivation of the invasive AC of *B. pertussis* in intact lymphocytes and in lymphocyte lysates requires ATP

In previous studies [5,13] we have shown that exposure of lymphocytes and erythrocytes to the invasive form of *B. pertussis* AC resulted in the accumulation of AC activity within these cells. The accumulation proceeded without any noticeable lag time, and reached a steady state within 10–30 min of incubation. Subsequent transfer of the cells into toxin-free medium led to a rapid loss of intracellular AC activity. We tested whether metabolic inhibitors would affect the penetration and inactivation of *B. pertussis* AC. Neither the glycolysis inhibitor, 2-deoxyglucose (50 mM), nor the inhibitor of oxidative phosphorylation, NaN_3 (10 mM), prevented the entry of the enzyme into human lymphocytes. However, treatment of the cells with both inhibitors together blocked 50–80% of *B. pertussis* AC penetration (results not shown). In contrast, the same treatment completely prevented the inactivation of the invasive AC within the cells (Fig. 1a). Transfer of cells into medium containing glucose and adenine restored the intracellular ATP level as well as the penetration and the inactivation of the invasive enzyme (results not shown). Inactivation of *B. pertussis* AC in lysates of lymphocytes pre-exposed to the invasive enzyme could be restored by addition of exogenous ATP (Fig. 1b). Preliminary studies showed that the inactivation of *B. pertussis* AC by lymphocyte lysates occurred in the presence of millimolar concentrations of ATP and MgCl_2 . However, under these conditions, and at the concentrations of *B. pertussis* AC used, the ATP was rapidly depleted due to the conversion of ATP to cAMP, and therefore the inactivation reaction was stopped. We have found that the inactivation of *B. pertussis* AC also occur in the presence of micromolar concentrations of free MgCl_2 and millimolar

of the partially purified invasive form of *B. pertussis* AC. After 20 min, cells were washed and cell lysates were prepared as described in the Experimental section. The lysates obtained were reincubated at 36 °C in the presence ($\circ$) or absence (Δ) of 2 mM-ATP. At the indicated times, three samples of 10 μ l each were withdrawn and AC activity was determined. 100% represents the activity of the enzyme at time zero of the inactivation reaction, which was 4.5 nmol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$. (c) The purified 200 kDa catalyst form of *B. pertussis* AC, with a specific activity of 0.34 nmol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$, was incubated in the absence ($\circ$) and presence ($\bullet$) of ATP with lysates of non-treated human lymphocytes, according to the procedure described in the Experimental section. At the indicated times the AC activity was determined. 100% represents the activity of the 200 kDa catalyst at time zero of the inactivation reaction.

Table 1. Inactivation of various forms of *B. pertussis* AC by lymphocyte lysates

The various forms of *B. pertussis* AC were obtained as described in the Experimental section. The inactivation of these forms was performed in the presence of ATP at 36 °C, using lymphocyte lysates as described in the Experimental section. 100% represents the activity of *B. pertussis* AC at zero time of the inactivation reaction.

Form of enzyme	AC activity remaining after 30 min inactivation (%)
Invasive	7.0
47 kDa	9.4
200 kDa	14.1
Crude extract	12.4

Fig. 2. Inactivation and degradation of ¹²⁵I-AC by lymphocyte lysates

(a) The 200 kDa form of *B. pertussis* AC was purified and iodinated as described in the Experimental section. The labelled catalyst was incubated with lymphocyte lysates

concentrations of uncomplexed ATP. Under these conditions, the conversion of ATP to cAMP during the inactivation reaction was only 5–10% of the total ATP added. The concentrations of ATP, MgCl₂, EDTA, CaCl₂ and CaM used in the inactivation reaction were 3 mM, 2 mM, 2 mM, 0.1 mM and 1 μM respectively. The concentrations of free MgCl₂, ATP–Mg complex and free ATP, calculated according to the Cation-Ligand binding program of Goldstein [24] were 15 μM, 0.25 mM and 2.7 mM respectively. The extent of inactivation was determined by quantification of AC activity in samples withdrawn from the inactivation at various times and diluted 50-fold into the AC assay mixture. The assay was performed for 5 min and the amount of cAMP produced was linear as a function of enzyme and time. The inactivation of the 200 kDa catalyst by lymphocyte lysates in the presence and absence of ATP is shown in Fig. 1(c). Table 1 demonstrates that all species of AC found in the bacterial extract (i.e. the 200 and 47 kDa catalysts and the invasive form [10]) were all inactivated by lymphocyte lysates in a similar fashion.

ATP-dependent degradation of ¹²⁵I-labelled *B. pertussis* AC

The 200 kDa catalyst purified from the invasive form of *B. pertussis* AC was iodinated according to the solid-phase procedure developed by Ladant [9]. The iodinated enzyme was catalytically active and responsive to CaM but lacked penetration capacity. The 200 kDa ¹²⁵I-labelled catalyst was incubated with lymphocyte lysates either in the absence or presence of ATP. Samples were withdrawn at various times and subjected to AC activity determinations and SDS/PAGE followed by autoradiography. Zero time samples were incubated on ice with lymphocyte lysates 5–10 min before AC determinations and PAGE. We found that incubation of the enzyme with the lysates in the presence of ATP led to its rapid inactivation, whereas incubation of the enzyme with the lysates in the absence of ATP increased its activity by 1.5–2-fold (Fig. 2a). This increase may be related to the decrease in the *B. pertussis* AC size. Rogel *et al.* [10] have found that the specific activity of the

either in the presence (●, ▲) or absence (○, △) of ATP. At the indicated times the AC activity was determined. The above was repeated in the presence (▲, △) or absence (●, ○) of the following protease inhibitors: PMSF (1 mM), leupeptin (2 mM), pepstatin (0.2 mM) and TPCK (0.1 mM). The lysates were pretreated for 1 h at 4 °C with 5 mM-*N*-ethylmaleimide, which was quenched with 5.5 mM-2-mercaptoethanol. 100% represents the activity of ¹²⁵I-labelled 200 kDa catalyst at time zero of the inactivation reaction. (b) Identical samples as in (a) were run on SDS/PAGE (9% gel) and the gel was autoradiographed as explained in the Experimental section. The left lane represents the ¹²⁵I-labelled 200 kDa catalyst which was incubated with ATP and CaM under the conditions of the inactivation reaction (see the Experimental section) but in the absence of lymphocyte lysates. The molecular mass standards with their masses in kDa were α-macroglobulin (180), β-galactosidase (116), fructose-6-phosphate kinase (84), pyruvate kinase (58), fumarate (48.5), lactate dehydrogenase (36.5) and triose-phosphate isomerase (26.5). The arrow indicates the position of the 42 kDa fragment which was generated in the absence of ATP.

Fig. 3. The 42 kDa fragment generated in the absence of ATP is associated with AC activity

^{125}I -labelled 200 kDa catalyst (200000 c.p.m.) was incubated at 36 °C for 30 min with lymphocyte lysates in the absence of ATP and then subjected to PAGE as explained in the Experimental section. The gel was cut into 0.4 cm slices, and each slice was placed into 0.5 ml of solution containing 2% CHAPS. Samples were assayed for AC activity (O) and the radioactivity was measured (●). The squares indicate the position of the molecular mass markers.

Table 2. Ability of various nucleotides to promote inactivation of *B. pertussis* AC by lymphocyte lysate

The inactivation was performed at 36 °C, using lymphocyte lysates and the 200 kDa form of *B. pertussis* AC, as described in the Experimental section. 100% represents the activity of *B. pertussis* AC at zero time of the inactivation reaction.

Nucleotide	AC activity remaining after 30 min inactivation (%)
None	101
ATP	11
GTP	97
CTP	98
GDP	123
ADP	123
AMP	181

47 kDa fragment of *B. pertussis* AC was higher than that of the 200 kDa form of the enzyme. Autoradiography revealed that both in the absence and the presence of ATP the enzyme was degraded (Fig. 2b). In the absence of ATP there was accumulation of a 42 kDa fragment which was resistant to further proteolysis (Fig. 2b). In the presence of ATP the enzyme was completely degraded but tiny amounts of the 42 kDa and other fragments could still be detected at zero time and after 5 min of incubation. A fragment of 160 kDa devoid of AC activity accumulated during degradation of the enzyme both in the presence and absence of ATP (Fig. 2b). In order to determine whether the 42 kDa fragment generated in the absence of ATP was endowed with adenylate cyclase

Fig. 4. Ability of various nucleotides to promote inactivation and degradation of *B. pertussis* AC

^{125}I -labelled AC was incubated at 36 °C for 40 min with lymphocyte lysates in the presence of the indicated nucleotides, at a final concentration of 3 mM. Then AC assay and autoradiography were performed as explained in the Experimental section. 100% represents the activity of the enzyme at time zero of the inactivation reaction.

activity, the gel was sliced and each slice was extracted with 2% (w/v) CHAPS and assayed for AC activity in the presence and absence of CaM. The peak of CaM-stimulated AC activity migrated with apparent molecular mass of 42 kDa and corresponded exactly with the migration of the peak of ^{125}I -labelled material, indicating that the 42 kDa fragment possesses both the catalytic and the CaM-binding sites (Fig. 3). The activities of the same fractions assayed in the absence of CaM were at least 20-fold lower.

Nucleotide specificity of the activation process

Several nucleotides were tested for their ability to support the proteolysis of *B. pertussis* AC. As shown in Table 2, neither AMP, ADP, GTP nor CTP promoted inactivation of *B. pertussis* AC. In contrast, App[NH]p could induce inactivation and degradation of *B. pertussis* AC to a similar extent to ATP (Fig. 4). In addition, both $\alpha\beta$ -methylene ATP and $\beta\gamma$ -methylene ATP promoted inactivation of the enzyme (results not shown). However, Gpp[NH]p, GTP[S] and cAMP did not support degradation or loss of enzyme activity, and the 42 kDa active fragment was generated as was observed in the control (Fig. 4). Since ATP analogues such as App[NH]p and $\beta\gamma$ -methylene ATP cannot support phosphorylation, and yet they induced inactivation and degradation of the enzyme, it is reasonable to assume that a phosphoenzyme intermediate is not involved in the process of enzyme degradation.

Table 3. Susceptibility of AC from various sources to inactivation by lymphocyte lysates

Crude dialysed urea extract of concentrated *B. pertussis* bacteria was prepared as described in [10]. Crude preparations of AC from *B. anthracis*, rat brain and human lymphocytes were prepared as described in the Experimental section. Human lymphocyte lysates were pretreated with 5 mM-*N*-ethylmaleimide as explained in the legend to Fig. 3(a). Leupeptin (2 mM), pepstatin (0.2 mM), PMSF (1 mM) and TPCK (0.1 mM) were included in the inactivation mixture to prevent possible non-specific proteolysis. The inactivation was performed at 36 °C and 100 % represents the activities of the various enzymes at zero time of inactivation. The specific activities of *B. pertussis*, *B. anthracis*, rat brain and human lymphocyte AC enzymes at zero time of the inactivation were $0.3 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, $0.7 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, $2 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ and $0.9 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ respectively.

Source of AC	AC activity remaining after 30 min inactivation (%)	
	– ATP	+ ATP
<i>B. pertussis</i>	185	10
<i>B. anthracis</i>	47	20
Rat brain	104	137
Human lymphocyte	84	89

Susceptibility of AC from other sources to inactivation and selectivity of the degradation reaction

To examine the susceptibility of other AC enzymes to inactivation we have incubated enzymes from various sources with lymphocyte lysates, in the presence and absence of ATP. As depicted in Table 3, neither a rat brain CaM-dependent AC nor the intrinsic lymphocyte AC were affected by the incubation. In contrast, *B. anthracis* AC, which is another calmodulin-dependent enzyme capable of entry into eukaryotic cells [25], lost 80 % of its activity in the presence of ATP. The inactivation of *B. anthracis* AC also occurred in the absence of ATP, but at a slower rate. Neither ^{125}I -labelled bovine serum albumin nor ^{125}I -human chorionic gonadotropin, which served as arbitrary controls, were degraded under these conditions. Protease inhibitors such as PMSF (1 mM), leupeptin (2 mM), pepstatin (0.2 mM) TPCK (0.1 mM) and *N*-ethylmaleimide (5 mM) did not inhibit the inactivation or prevent the degradation of *B. pertussis* AC (Fig. 2). However, the inactivation could be blocked by heating the lysates at 80 °C for 5 min before addition of the enzyme. The rate of degradation of ^{125}I -labelled *B. pertussis* AC by lymphocyte lysates was not slowed down by the presence of bovine serum albumin ($1 \text{ mg} \cdot \text{ml}^{-1}$), thus indicating that the corresponding protease can single out minute quantities of *B. pertussis* AC.

DISCUSSION

The data presented in this study clearly demonstrate that lymphocyte lysates possess proteolytic activity which degrades and inactivates all forms of *B. pertussis* AC. The 200 kDa catalyst of the enzyme (isolated from the invasive form) is cleaved by lymphocyte lysates and generates an active fragment of 42 kDa. This fragment is

completely degraded and inactivated in the presence of millimolar concentrations of exogenous ATP. It appears that the degradation of the 200 kDa catalyst in the presence and the absence of ATP is conducted by the same protease(s). This conclusion is supported by the facts that (1) similar fragments are produced during degradation of the enzyme in the presence and absence of ATP (Figs. 2 and 4), and (2) both degradation processes are unaffected by the same protease inhibitors or the presence of eukaryotic proteins. The notion that the degradation and inactivation of *B. pertussis* AC by lymphocyte lysates is related to the inactivation of the invasive enzyme observed within intact cells is suggested by the following findings. Depletion of ATP by metabolic inhibitors prevents the inactivation of the invasive AC in intact human lymphocytes and addition of exogenous ATP to lymphocyte lysates restores it. In addition, the kinetics of the enzyme inactivation both in lysates and intact cells fit a first-order reaction with half-life of 8–15 min. Direct demonstration that the invasive enzyme is inactivated by proteolysis in intact cells must wait until an invasive and labelled preparation of *B. pertussis* AC is available.

The mechanism by which ATP promotes inactivation of *B. pertussis* AC is not known. It is tempting to hypothesize that ATP or ATP analogues bind to *B. pertussis* AC rendering the 42 kDa polypeptide susceptible to complete degradation, rather than acting by stimulation of an ATP-dependent protease. This hypothesis is supported by the following arguments. Usually, breakdown of proteins in mammalian and bacterial cells by ATP-dependent proteases requires hydrolysis of ATP and the presence of millimolar concentrations of free Mg^{2+} [26,27]. In the case of *B. pertussis* AC, the degradation occurs in the presence of an excess of uncomplexed ATP and micromolar concentrations of free MgCl_2 . Under these conditions, the nucleotide is bound to the catalytic sites of AC but the production of cAMP is inhibited [28]. Furthermore, non-hydrolysable analogues of ATP such App[NH]_p, and $\beta\gamma$ -methylene ATP promote the inactivation and degradation of the enzyme at similar efficiency as ATP. $\alpha\beta$ -Methylene ATP, an analogue which is not converted to cAMP but is known to competitively inhibit AC activity [29], promotes the inactivation of *B. pertussis* AC. *N*-Ethylmaleimide, which blocks ubiquitin-mediated proteolysis of proteins [30], has no effect on the degradation of *B. pertussis* AC.

Although both *B. pertussis* and *B. anthracis* produce AC toxins with shared features, including activation by CaM and the ability to invade target cells, it appears that the anthrax AC enters cells by receptor-mediated endocytosis rather than by direct penetration of the plasma membrane [31]. Nevertheless, when anthrax-treated Chinese hamster ovary cells are placed in AC-free medium, a rapid decrease in level of cAMP is observed, suggesting that the internalized *B. anthracis* AC is unstable within the cytoplasm of the target cell [25]. We have shown that incubation of a crude preparation of *B. anthracis* AC with lymphocyte lysates led to a decay of enzyme activity which was enhanced by ATP. It is thus tempting to speculate that the same protease(s) found in the target cells is responsible for the inactivation of both types of invasive enzymes. Degradation of proteins in eukaryotic cells can be carried out by several different pathways [32]. Most of the work on protein breakdown

has been performed either by using cell-free preparations [30] or by microinjection of radiolabelled proteins [33]. Although the exact pathway of *B. pertussis* AC degradation in the host has not been completely clarified, the study of *B. pertussis* inactivation has obvious advantages. It represents a system in which a toxic protein penetrates eukaryotic target cells wherein it is specifically inactivated and degraded. The structure of this protease, its cellular localization and its role in the inactivation of other toxins remain to be elucidated.

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