INFECTION AND IMMUNITY, June 1989, p. 1725–1730
0019-9567/89/061725-06$02.00/0

Vol. 57, No. 6

Dissociation of Catalytic and Invasive Activities of *Bordetella pertussis* Adenylate Cyclase

ANASTASSIOS RAPTIS, LESLIE KNIPLING, AND J. WOLFF*

National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Maryland 20892

Received 19 December 1988/Accepted 22 February 1989

Bordetella pertussis **organisms secrete adenylate cyclase, at least one form of which can invade host cells and appears to be a virulence factor. Treatment of urea extracts containing invasive cyclase of *B. pertussis* with trypsin, chymotrypsin, or subtilisin abolishes the ability to increase intracellular cyclic AMP levels in CHO cells (invasiveness) at concentrations that have minimal or no effects on adenylate cyclase activity. Higher protease concentrations can inhibit catalytic activity, and 1 μM calmodulin protects this catalytic activity, but not invasiveness, against proteolytic inhibition. Rabbit immunoglobulin G (IgG) fractions from antisera prepared against urea extracts inhibited invasiveness at 10-fold-lower concentrations than inhibited catalytic activity. One IgG from a rabbit immunized against a partially purified, noninvasive form of the *B. pertussis* adenylate cyclase inhibited catalytic activity but was ineffective against invasiveness. We conclude that these two properties of the adenylate cyclase are independent functions that reside on different domains of the same protein or on different proteins.**

The extracellular adenylate cyclase of virulent *Bordetella* species is believed to be a virulence factor on the basis of the following arguments. (i) The cyclase can raise cyclic AMP (cAMP) levels of many cells far beyond what can be achieved with stimulation of the cells' own adenylate cyclase (even when activated by other bacterial toxins such as cholera or pertussis toxin) (11, 27). Macrophages attain cAMP levels high enough to paralyze those functions that would be effective in combating the infection (7, 9, 14). (ii) *B. pertussis* mutants lacking adenylate cyclase are avirulent (35). Similarly, degraded strains that have lost their virulence no longer contain significant levels of the calmodulin-activated adenylate cyclase (6, 16, 34). (iii) *Bordetella* species in which the pertussis toxin gene is not expressed (1) (*B. parapertussis* and *B. bronchoseptica*) retain virulence and secrete an active, calmodulin-requiring enzyme that generally resembles that from *B. pertussis* (8, 28).

The enzyme requires calmodulin for activity (36). A pure form of the enzyme has been identified as a protein of ca. 45 to 47 kilodaltons (kDa) (21, 25, 37), but larger forms have also been shown to occur (14, 20, 30; unpublished observations), and a 190-kDa form has been cloned in *Escherichia coli* (5, 12). The cloned 190-kDa form has not yet been successfully tested for invasiveness, and large forms prepared directly from *B. pertussis* cultures have not been sufficiently purified to differentiate tight complexes from single invasive proteins (30). As an approach to relating the invasive to the catalytic properties of *B. pertussis* adenylate cyclase, we have used limited proteolysis and inhibition by antibodies to separate these functions.

MATERIALS AND METHODS

Invasive adenylate cyclase of *B. pertussis* 114 was prepared by urea extraction of 24-h growths as described previously (10). DEAE chromatography of these extracts yielded a noninvasive form (10) with specific activities of 0.6 to 4.3 μmol of cAMP/min per mg. Adenylate cyclase was measured at 30°C for 10 min in a total volume of 60 μl containing 60 mM Tris hydrochloride buffer (pH 7.9), 2 mM $MgCl_2$, 1 mM ATP, ≈0.5 μCi of [α-^{32}P]ATP, 10 μM added $CaCl_2$, and 1.0 or 0.1 μM bovine brain calmodulin, and [^{32}P]cAMP levels were determined (10). Protein was assayed by the method of Bradford for column profiles (3); otherwise it was determined by the bicinchoninic acid method (32) with bovine serum albumin standards.

Chinese hamster ovary (CHO) cells were grown in 12-well trays in Eagle minimal essential medium fortified with nonessential amino acids, 10% fetal calf serum, glutamine, penicillin, and streptomycin. Cells were washed once with protein-free medium for 15 min, incubated at 37°C with the cyclase in 1.0 ml of medium containing 0.3 mM isobutylmethylxanthine (final ethanol concentration, 1%) for 60 min, washed twice in cold serum-free medium, and immediately frozen on dry ice. When immunoglobulin G (IgG) was tested, cells were incubated for 15 min at 37°C before addition of cyclase. cAMP was assayed by radioimmunoassay as described previously (29).

Conditions for proteolysis were determined from time and concentration curves at different enzyme-urea extract weight ratios for trypsin, chymotrypsin, and subtilisin. All reactions were carried out in 20 mM Tris hydrochloride buffer, pH 7.5, on ice for 10 min or as indicated. Trypsin and chymotrypsin reactions were stopped with 50 μM aprotinin or at constant weight ratios, and subtilisin reactions were stopped with fresh 0.5 mM phenylmethylsulfonyl fluoride in dimethyl sulfoxide. When 1 μM bovine brain calmodulin was used as a protector, an equivalent weight (17 μg/ml) of bovine serum albumin served as a control and samples were diluted 1:50 before assay. In all cases, one of at least three experiments is reported.

Rabbits (strain P2W, 7 weeks of age, weighing ≈1 kg) were injected intradermally with 155 μg of a partially purified form of the *B. pertussis* adenylate cyclase in complete Freund adjuvant and boosted three times. Another set of rabbits were injected with 1.6 mg of urea extract in complete Freund adjuvant and boosted three times intradermally and two times intravenously with 546 μg of urea extract. In some rabbits, subcutaneous glucose injections were necessary because of transient hypoglycemia (15). Sera were precipitated with 50% saturated ammonium sulfate over 6 to 8 h at

* Corresponding author.

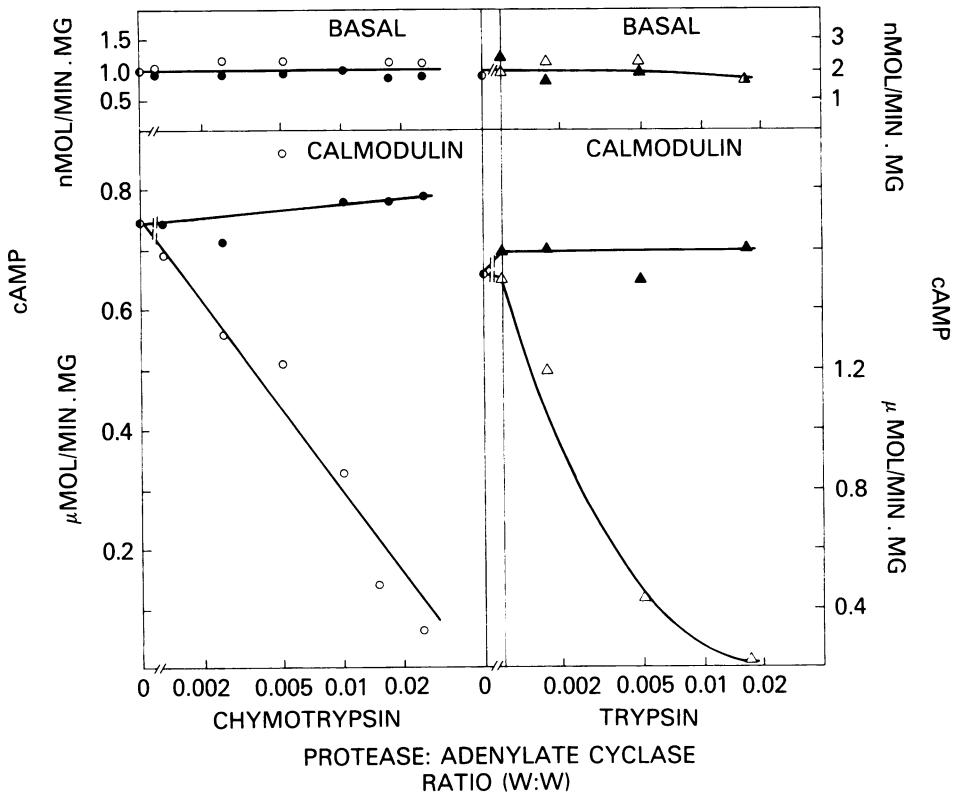

FIG. 1. Inhibition of basal and calmodulin-stimulated adenylate cyclase activity by chymotrypsin and trypsin. Urea extract adenylate cyclase (140 μg) was exposed on ice at pH 7.5 for 10 min to either chymotrypsin (left panels) or trypsin (right panels) at the ratios indicated. Digestion was stopped by the addition of a 200-fold molar excess of aprotinin. Inactivated protease was used as a control. All values are means of triplicates. Solid symbols, Controls; open symbols, treated with protease; circles, chymotrypsin; triangles, trypsin; half-solid circles, adenylate cyclase and aprotinin controls.

4°C, pellets were dissolved with H_2O, and the ammonium sulfate precipitation was repeated twice. The dissolved pellets were dialyzed in 0.0175 M $NaPO_4$ buffer, pH 6.3, centrifuged briefly, and run over DE 52 columns, previously equilibrated and eluted with the same buffer. The protein content was measured, and the flowthrough peak tubes were pooled, dialyzed against 0.025 M Tris hydrochloride buffer (pH 7.9), and then lyophilized (rabbits 1, 2, and 4) or lyophilized and then dialyzed (rabbits 6, 7, and 8).

Reagents used were as follows: trypsin-toluene sulfonyl phenylalanyl chloromethyl ketone was from Worthington Biochemicals; chymotrypsin type VII, subtilisin, and phenylmethylsulfonyl fluoride were from Sigma Chemical Co., and aprotinin was from Boehringer Mannheim. All other reagents were obtained as described previously (10).

For the protection experiment shown in Table 1, crude adenylate cyclase (613 μg) was incubated with either 1 μM calmodulin or 1 mM ATP for 5 min at room temperature and cooled on ice. Trypsin was added at a ratio of 1:1,200 (wt/wt) (2.6 μg/ml) and chymotrypsin at 1:1,000 (3.1 μg/ml) and incubated on ice for 10 min at pH 7.5. Preincubated trypsin was used as a control. Washed CHO cells were exposed to adenylate cyclase alone (281 nmol/min per ml) or to adenylate cyclase treated for 60 min at 37°C, and intracellular cAMP levels were determined. Results are means of triplicate determinations. For cyclase assays, adenylate cyclase (100 μg) was incubated with either 1 μM calmodulin or 1 mM ATP for 5 min at room temperature and cooled on ice. Trypsin was then added at 1:400 (5.3 μg/ml) and chymotrypsin at 1:200 (10.6 μg/ml) and incubated for 10 min at pH 7.5 on ice. Samples were diluted 1:40 into 0.1% serum albumin (pH 7.9) and assayed for cyclase activity in triplicate.

RESULTS

Effect of proteolysis on catalytic and invasive properties. The catalytic activity was known to be trypsin sensitive (17), and more detailed studies showed considerable sensitivity of the cyclase of urea extracts to three different proteases: trypsin, chymotrypsin, and subtilisin. Basal and calmodulin-stimulated adenylate cyclase activities were affected by chymotrypsin and trypsin (Fig. 1). The weight ratios correspond to chymotrypsin concentrations of 4.0 to 77.8 μg/ml and trypsin concentrations of 4.1 to 51.8 μg/ml. To obtain slow enough rates, reactions were carried out at 4°C and for only 10 min. Although phenylmethylsulfonyl fluoride was a less satisfactory inhibitor than aprotinin, similar results were obtained for both trypsin and chymotrypsin when proteolysis was stopped with this agent.

We also tested subtilisin because of its broad specificity. Subtilisin proved to be more difficult to work with because even at 0.5 mM phenylmethylsulfonyl fluoride, there remained some residual proteolytic activity when more than 1:1,000 ratios of protease to protein were used (this effect was primarily on invasiveness). Nevertheless, at a weight ratio of 1:2,000, substantial inhibition of invasiveness could be produced under conditions in which there was little effect on adenylate cyclase activity (data not shown). It is clear, therefore, that with three different proteases, the invasive properties of the adenylate cyclase preparation are consid-

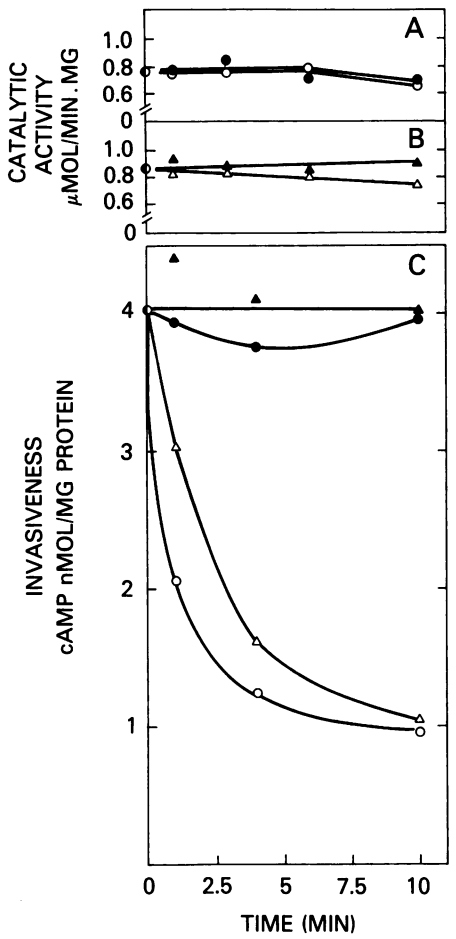

FIG. 2. Comparison of the sensitivity of the catalytic and invasive properties of *B. pertussis* adenylate cyclase to proteolysis. Urea extract adenylate cyclase (612 μg) was exposed on ice at pH 7.5 to chymotrypsin at 1:1,000 (0.61 μg) or trypsin at 1:1,200 (0.51 μg). Digestion was stopped by 50 μM aprotinin. Proteases were inactivated for the same time and used as controls. Washed CHO cells were incubated for 60 min with 281 nmol/min per ml of intact or proteolyzed cyclase and assayed for cAMP (20) (panel C). Values are means of triplicate determinations. For catalytic activity, the preparations were diluted 1:50 in 20 mM Tris (pH 7.9)–0.1% bovine serum albumin and assayed for [^{32}P]cAMP production (10). (A) Chymotrypsin; (B) trypsin. Symbols: ●, ▲, controls (inactivated protease); ○, △, experimental conditions; ◐, adenylate cyclase alone and aprotinin; ○, ●, chymotrypsin; △, ▲, trypsin.

erably more susceptible to proteolysis than is the catalytic activity.

To check effects of chymotrypsin and trypsin on invasiveness, it was obviously necessary to use even lower protease-urea extract ratios to detect differential effects, if any. We thus chose low concentrations of protease and 10-min incubations at 4°C, followed by addition of 50 μM aprotinin to trypsin or chymotrypsin as our standard proteolysis conditions, and we compared the effect of such treatment on the catalytic and invasive activities of the adenylate cyclase. As shown in Fig. 2, concentrations of trypsin were found by trial and error (1:1,200, or 2.6 μg/ml) that had negligible effects on catalytic activity but virtually abolished invasion of CHO cells by the adenylate cyclase. From such time curves, we found that the ability of adenylate cyclase to penetrate CHO cells is many times more sensitive to trypsin

TABLE 1. Selective protection by calmodulin of catalytic activity against trypsin and chymotrypsin

Protease	Addition	Catalytic activity (μmol of cAMP/min per mg)		Invasiveness (nmol of cAMP/min per mg)	
		Control	Experimental[a]	Control	Experimental[b]
Trypsin	None	1.12	0.44	2.3	0.94
	Calmodulin (1 μM)	0.92	1.76	2.2	0.53
	ATP (1 mM)	1.12	0.56	—[c]	—
Chymo-trypsin	None	1.08	0.60	2.2	0.16
	Calmodulin (1 μM)	1.39	1.32	1.4	0.10
	ATP (1 mM)	1.04	0.68	—	—

[a] Dilutions were 1:400 and 1:200 for trypsin and chymotrypsin, respectively.
[b] Dilutions were 1:1,200 and 1:1,000 for trypsin and chymotrypsin, respectively.
[c] —, Not determined.

than the catalytic activity. Exact estimates are, however, not possible because of the very slow proteolysis of the adenylate cyclase activities.

Equally marked differences could be demonstrated with chymotrypsin. Under the same incubation conditions and a weight ratio of chymotrypsin to cyclase of 1:1,000 (3.1 μg/ml), there was complete dissociation of the proteolytic effect on invasiveness of CHO cells (cAMP accumulation) and catalytic activity (Fig. 2, open circles), and the difference in susceptibilities to this protease was even greater than for trypsin.

Protection by calmodulin. Calmodulin is a potent activator of *B. pertussis* adenylate cyclase (36), and thus the possibility that it might protect the enzyme against proteolysis merited consideration. Adenylate cyclase was incubated with 1 μM bovine brain calmodulin (17 μg/ml) or an equivalent weight of bovine serum albumin at room temperature for 5 min and subsequently exposed to higher concentrations of trypsin (1:400 [5.3 μg/ml]) or chymotrypsin (1:200 [10.6 μg/ml]) for 10 min at 4°C, after which proteolysis was stopped with aprotinin. The preparation was diluted 1:50 for assay of adenylate cyclase activity.

Calmodulin pretreatment protected the catalytic activity against trypsin and chymotrypsin (Table 1). This was not a nonspecific protein effect, because an equivalent mass of bovine serum albumin failed to prevent proteolysis of the cyclase. By contrast, calmodulin did not protect invasiveness against the action of these proteases (Table 1). The apparent increase in trypsin susceptibility in the presence of calmodulin (Table 1) was not consistently observed. cAMP accumulation in CHO cells was inhibited ≈70% whether calmodulin was present or not. Note that lower protease concentrations had to be used for this assay. Calmodulin protection thus constitutes a second example of dissociation of catalytic from invasive effects of the cyclase preparation.

An interesting side effect of the proteolysis of adenylate cyclase preparations by trypsin in the presence of calmodulin was the enhancement of catalytic activity (Table 1). This was always seen in these experiments and amounted to an average of 40% in five experiments. Chymotrypsin led to a smaller or negligible stimulation. Whether this is the result of the removal of an inhibitor or results from a proteolytic cut not possible or less favored in the uncomplexed cyclase remains to be determined. Such activation is known to occur with some other calmodulin-activated enzymes.

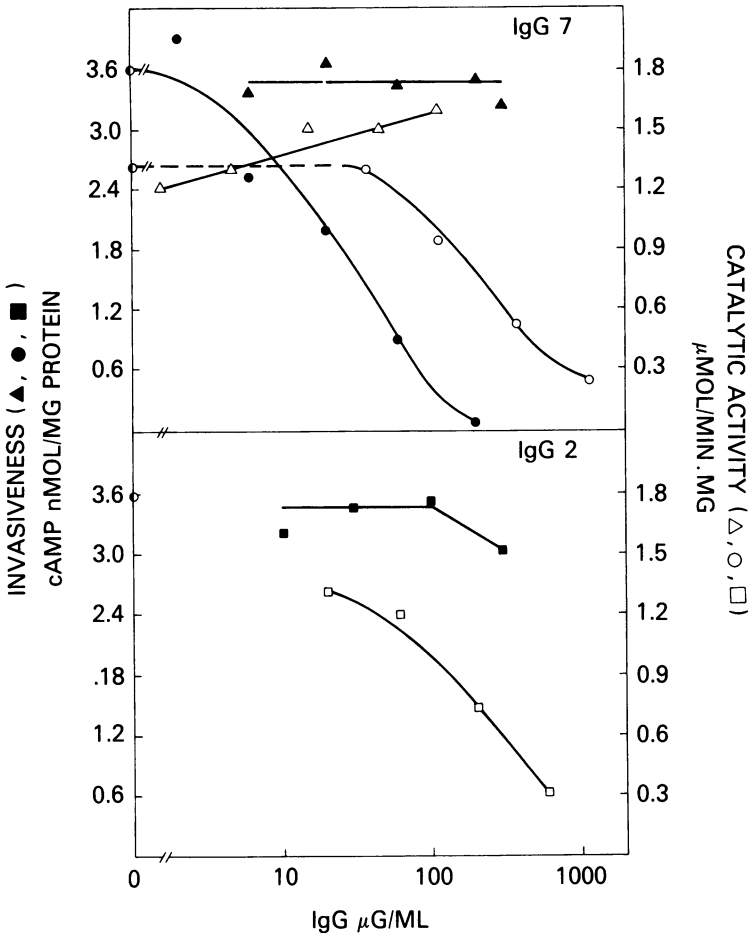

FIG. 3. Differential inhibition of invasiveness and catalytic activity of adenylate cyclase by anti-adenylate cyclase antibodies. CHO cells were incubated for 15 min with either IgG-7 or IgG-2 at 37°C. Adenylate cyclase (222 nmol/min per ml) was then added, incubation was continued for 60 min, and intracellular cAMP was determined (29). The values are means of triplicate determinations. For cyclase assays, IgG-7 was added to adenylate cyclase at 4°C for 60 min, brought to 30°C for 2 min, and assayed. All values are means of triplicate determinations. Open symbols, Catalytic activity; solid symbols, invasiveness; half-solid circles, adenylate cyclase alone; triangles, commercial IgG.

The possibility that proteolysis occurred at the calmodulin-binding site rather than at the catalytic site had to be considered. To shed light on this, the effect of trypsin on basal adenylate cyclase activity (assayed in the absence of any calmodulin) was measured at the same trypsin-cyclase weight ratio during proteolysis but with 10 times as much enzyme for the assay. Trypsin and chymotrypsin concentrations that completely inhibited calmodulin-stimulated activity did not inhibit basal adenylate cyclase activity (Fig. 2A and B). These data suggest that proteases may inhibit the calmodulin-stimulated catalytic activity primarily by abolishing the binding of calmodulin. This has been found previously under more drastic conditions of proteolysis (21). The fact that ATP, which would be expected to shield the catalytic site, failed to protect the cyclase against trypsin or chymotrypsin is consistent with such a postulate (Table 1).

Differential inhibition by polyclonal antibodies. Polyclonal antibodies were produced in rabbits against either the urea extracts of *B. pertussis* (strain 114) or a DEAE-purified preparation. Because of numerous inhibitory factors present in serum, it was necessary to purify these sera to the IgG stage to facilitate comparisons. All six IgG fractions completely inhibited calmodulin-stimulated catalytic activity of the cyclase preparations with a 50% inhibitory concentration of 150 to 300 μg of IgG per ml (data not shown). Preincubation of the enzyme with IgG from 10 to 60 min increased potency by a factor of two to three. The dose-response curve of one such IgG preparation is depicted in Fig. 3 (top panel). Control IgG preparations had no effect (triangles). The same IgG preparation inhibited invasiveness with ≈10-fold greater potency. Other IgG preparations from sera prepared against urea extracts inhibited invasiveness at a three- to fourfold lower concentration than catalytic activity.

One IgG preparation obtained from a rabbit immunized against a DEAE-purified, noninvasive form of the cyclase exhibited anticatalytic potency like the other sera but had virtually no antiinvasive effect in CHO cells exposed to an invasive form of the enzyme (Fig. 3, bottom panel). These findings provide a third example of the dissociation of invasiveness and catalytic activity and suggest that different epitopes are involved in the inhibition of the catalytic and invasive properties of the cyclase.

DISCUSSION

A considerable number of microbial toxins act through regulation of cAMP levels of host cells. These include toxins from *Vibrio cholerae* (27), *Escherichia coli* (27), *Bacillus*

subtilis (19), *Yersinia pestis* (2), *Kluyveromyces lactis* (33), *Bordetella pertussis* and related species (18), and *Bacillus anthracis* (23). In the majority of examples, the toxins interact with the adenylate cyclase-cAMP system of the host cells. With *Bordetella* species and *Bacillus anthracis*, however, a highly active, calmodulin-requiring adenylate cyclase is provided by the microorganism. The pertussis cyclase can be clearly distinguished from pertussis toxin by causing much greater cAMP accumulation, failure to be neutralized by anti-pertussis toxin antibodies, and more rapid onset of cAMP accumulation.

The facts that the *B. pertussis* adenylate cyclase appears to be a virulence factor (see above) and has a largely extracellular location (17) suggested that penetration of the host cell plasma membrane might be required. Indirect evidence for this stems from the findings that cells exposed to the invasive form of the enzyme contain cyclase activity different from that of the host (9), that a fraction of this enzyme cannot be washed off (9, 11), and that the enzyme can generate cAMP in human erythrocytes, which contain trivial amounts of their own cyclase (11, 14, 31). Penetration does not occur by endocytosis (11, 13, 14), but the method of translocation has not been elucidated. For the cyclase from *B. anthracis*, the evidence is consistent with a binary toxin model in which the binding moiety is a separate protein, the protective antigen. This factor, with the help of a small proteolytic cut, translocates the catalytic moiety (edema factor) to the interior (23, 24). No similar mechanism has yet been shown for the functionally similar but physically different adenylate cyclase from *B. pertussis*. However, the present results suggest that such a mechanism is involved, because translocation or invasiveness is readily dissociated from catalytic activity on a number of operational grounds, such as sensitivity to proteolysis, protection against proteolysis by calmodulin, and sensitivity to antibodies. Moreover, the marked sensitivity of *Bordetella* adenylate cyclase preparations to proteolysis probably accounts for many of the discrepant results reported in the literature regarding size and invasiveness (12, 14, 18, 20, 21, 25, 37).

The catalytic and calmodulin-binding sites are believed to reside in the N-terminal end of the ≈190-kDa adenylate cyclase (with a 5.2-kilobase open reading frame encoding a 1,706-amino-acid polypeptide) (12). It was of interest, therefore, to test for possible protection of the enzyme by calmodulin occupancy. Calmodulin provided virtually complete protection of adenylate cyclase activity against inactivation by trypsin (Table 1), and similar results have been reported recently under very different conditions of proteolysis (21). Calmodulin also provided protection against chymotrypsin (Table 1) and subtilisin (data not shown). These results do not distinguish between inability to activate the catalytic activity with calmodulin from damage to the catalytic site. Our preliminary results with basal activity suggest that the catalytic site remains intact; hence, the former of these mechanisms seems the more probable. This is confirmed by the fact that ATP did not protect the catalytic activity against proteolysis (Table 1). The invasive activity of these *Bordetella* preparations was not protected by 1 μM calmodulin, suggesting that this domain was separate, remote from the calmodulin-binding site, or not under conformational control by the regulator.

Polyclonal or monoclonal antibodies prepared against *B. pertussis* adenylate cyclase inhibit catalytic activity or interact with several forms of the enzyme (4, 21, 26, 30), and we have shown that U.S. standard antipertussis rabbit antisera (11) and hyperimmune human sera (generously provided by C. R. Manclark, Food and Drug Administration) cause near total inhibition of *B. pertussis* adenylate cyclase activity (manuscript in preparation). Polyclonal antibodies prepared in rabbits against urea extracts of *B. pertussis* (strain 114) organisms readily inhibit catalytic activity, but titers against invasive activity are higher than against catalytic activity, and an antiserum prepared against a noninvasive form of the enzyme had normal anticatalytic potency but was virtually devoid of antiinvasive activity.

Together, these data suggest that the protein domains involved in invasiveness and catalytic activity are different. Two possible modes of invasion by the cyclase are visualized. In one, a single gene product (which may need to be modified posttranslationally to produce the invasive form) is subsequently hydrolyzed, with loss of invasiveness but retention of catalytic activity. From the sequence of the cloned activities (5, 12), the penetration domain would have to be "downstream" (toward the C terminus) from the catalytic domain. Because a second gene product or use of a degraded enzyme cannot be ruled out, the present data are equally compatible with a binary toxin in which a separate invasive factor is more sensitive to proteolysis than the catalytic unit. The possibility that such a factor may exist was recently proposed (M. G. Donovan, H. R. Masure, and D. R. Storm, abstr. 2395, J. Cell Biol. **107**:420a, 1988). A decision between these two modes will require more highly purified factors.

ACKNOWLEDGMENTS

We thank Charles Manclark, Van Sickler, and Julie Hannah for continued help in growing the *B. pertussis* organisms and V. R. deGrange for editorial assistance and manuscript preparation.

LITERATURE CITED

1. **Arico, B., and R. Rappuoli.** 1987. *Bordetella parapertussis* and *Bordetella bronchoseptica* contain transcriptionally silent pertussis toxin genes. J. Bacteriol. **169**:2847–2853.
2. **Aseeva, L. E., L. A. Shevchenko, N. I. Shimaniuk, B. D. Rublev, and B. N. Mishankin.** 1987. Assessment of the modulating effect of adenylate cyclase of the plague microbe on guinea pig peritoneal leukocytes with the aid of chemiluminescence. Zh. Mikrobiol. Epidemiol. Immunobiol. **7**:59–63.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
4. **Brezin, C., N. Guiso, D. Ladant, L. Djavadi-Ohaniance, F. Megret, I. Onyeocha, and J. M. Alonso.** 1987. Protective effects of anti *Bordetella pertussis* adenylate cyclase antibodies against lethal respiratory infection of the mouse. FEMS Microbiol. Lett. **42**:75–80.
5. **Brownlie, R. M., J. G. Coote, R. Parton, J. E. Schultz, A. Rogel, and E. Hanski.** 1988. Cloning of the adenylate cyclase genetic determinant of *B. pertussis* and its expression in *E. coli* and *B. pertussis*. Microb. Pathogenesis **4**:335–344.
6. **Brownlie, R. M., R. Parton, and J. G. Coote.** 1985. The effect of growth conditions on adenylate cyclase activity and virulence-related properties of *Bordetella pertussis*. J. Gen. Microbiol. **13**:27–38.
7. **Confer, D. L., and J. W. Eaton.** 1982. Phagocyte impotence caused by an invasive bacterial adenylate cyclase. Science **217**:948–950.
8. **Endoh, M., T. Takezawa, and Y. Nakase.** 1980. Adenylate cyclase activity of *Bordetella* organisms. Microbiol. Immunol. **24**:95–104.
9. **Friedman, E., Z. Farfel, and E. Hanski.** 1987. The invasive adenylate cyclase of *Bordetella pertussis*. Biochem. J. **243**: 145–151.
10. **Gentile, F., A. Raptis, L. G. Knipling, and J. Wolff.** 1988. Extracellular cAMP formation from host cell ATP by *Bordetella*

pertussis adenylate cyclase. Biochim. Biophys. Acta **971**:63–71.
11. **Gentile, F., A. Raptis, L. G. Knipling, and J. Wolff.** 1988. *Bordetella pertussis* adenylate cyclase—penetration into host cells. Eur. J. Biochem. **175**:447–453.
12. **Glaser, P., D. Ladant, O. Sezer, F. Pichot, A. Ullmann, and A. Danchin.** 1988. The calmodulin-sensitive adenylate cyclase of *Bordetella pertussis*: cloning and expression of *E. coli*. Mol. Microbiol. **2**:19–30.
13. **Gordon, V. M., S. H. Leppla, and E. L. Hewlett.** 1988. Inhibitors of receptor-mediated endocytosis block the entry of *Bacillus anthracis* adenylate cyclase toxin but not that of *Bordetella pertussis* adenylate cyclase toxin. Infect. Immun. **56**:1066–1069.
14. **Hanski, E., and Z. Farfel.** 1985. *Bordetella pertussis* invasive adenylate cyclase. J. Biol. Chem. **260**:5526–5532.
15. **Hewlett, E. L., C. O. Roberts, J. Wolff, and C. R. Manclark.** 1983. Biphasic effect of pertussis vaccine on serum insulin in mice. Infect. Immun. **41**:137–144.
16. **Hewlett, E. L., L. H. Underhill, S. A. Vargo, J. Wolff, and C. R. Manclark.** 1978. *Bordetella pertussis* adenylate cyclase: regulation of activity and its loss in degraded strains, p. 81–85. *In* C. R. Manclark and J. C. Hill (ed.), International Symposium on Pertussis. U.S. Department of Health, Education, and Welfare publication NIH-79-1830. National Institutes of Health, Bethesda, Md.
17. **Hewlett, E. L., M. A. Urban, C. R. Manclark, and J. Wolff.** 1976. Extracytoplasmic adenylate cyclase of *Bordetella pertussis*. Proc. Natl. Acad. Sci. USA **73**:1926–1930.
18. **Hewlett, E., and J. Wolff.** 1976. Soluble adenylate cyclase from the culture medium of *Bordetella pertussis*: purification and characterization. J. Bacteriol. **127**:890–898.
19. **Hosono, K., and H. Suzuki.** 1985. Morphological transformation of CHO cells by arylpeptides, inhibitors of cAMP PDE, produced by *B. subtilis*. J. Biol. Chem. **260**:11252–11255.
20. **Kessin, R. H., and J. Franke.** 1986. Secreted adenylate cyclase of *Bordetella pertussis*: calmodulin requirements and partial purification of two forms. J. Bacteriol. **166**:290–296.
21. **Ladant, D.** 1988. Interaction of *Bordetella pertussis* adenylate cyclase with calmodulin. J. Biol. Chem. **263**:2612–2618.
22. **Ladant, D., C. Brezin, J. M. Alonso, I. Crenon, and N. Guiso.** 1986. *Bordetella pertussis* adenylate cyclase. J. Biol. Chem. **261**:16264–16269.
23. **Leppla, S. H.** 1982. Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cAMP concentrations in eukaryotic cells. Proc. Natl. Acad. Sci. USA **79**:3162–3166.
24. **Leppla, S. H.** 1984. *Bacillus anthracis* calmodulin-dependent adenylate cyclase. Adv. Cyclic Nucleotide Res. **17**:189–198.
25. **Masure, H. R., D. L. Oldenburg, M. G. Donovan, R. L. Shattuck, and D. R. Storm.** 1988. The interaction of Ca^{2+} with the calmodulin-sensitive adenylate cyclase from *Bordetella pertussis*. J. Biol. Chem. **263**:6933–6940.
26. **Monneron, A., D. Ladant, J. d'Alayer, J. Bellalou, O. Barzu, and A. Ullmann.** 1988. Immunological relatedness between *Bordetella pertussis* and rat brain adenylate cyclases. Biochemistry **27**:536–539.
27. **Moss, J., D. Burns, J. A. Hsia, E. L. Hewlett, R. L. Guerrant, and M. Vaughan.** 1984. Cyclic nucleotides: mediators of bacterial toxin action in disease. Ann. Intern. Med. **101**:653–666.
28. **Pearson, R. D., P. Symes, M. Conboy, A. Weiss, and E. L. Hewlett.** 1987. Inhibition of monocyte oxidative responses by *Bordetella pertussis* adenylate cyclase toxin. J. Immunol. **139**: 2749–2754.
29. **Raptis, A., L. G. Knipling, F. Gentile, and J. Wolff.** 1989. Modulation of invasiveness and catalytic activity of *Bordetella pertussis* adenylate cyclase by polycations. Infect. Immun. **57**:1066–1071.
30. **Rogel, A., Z. Farfel, S. Goldschmidt, J. Shiloach, and E. Hanski.** 1988. *Bordetella pertussis* adenylate cyclase. J. Biol. Chem. **263**:13310–13316.
31. **Shattuck, R. L., and D. R. Storm.** 1985. Calmodulin inhibits entry of *Bordetella pertussis* adenylate cyclase into animal cells. Biochemistry **24**:6323–6328.
32. **Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk.** 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. **150**:76–85.
33. **Sugisaki, Y., N. Gunge, K. Sakaguchi, M. Yamasaki, and G. Tamura.** 1983. *Kluyveromyces lactis* killer toxin inhibits adenylate cyclase of sensitive yeast cells. Nature (London) **304**: 464–466.
34. **Weiss, A. A., and S. Falkow.** 1984. Genetic analysis of phase change in *Bordetella pertussis*. Infect. Immun. **43**:263–269.
35. **Weiss, A. A., E. L. Hewlett, G. A. Myers, and S. Falkow.** 1983. Transposon Tn*5*-induced mutations affecting virulence factors of *Bordetella pertussis*. Infect. Immun. **42**:33–45.
36. **Wolff, J., G. H. Cook, A. R. Goldhammer, and S. A. Berkowitz.** 1980. Calmodulin activates prokaryotic adenylate cyclase. Proc. Natl. Acad. Sci. USA **77**:3840–3844.
37. **Wolff, J., G. H. Cook, A. R. Goldhammer, C. Londos, and E. L. Hewlett.** 1984. *Bordetella pertussis*: multiple attacks on host cell cAMP regulation. Adv. Cyclic Nucleotide Res. **17**:161–172.