Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911–917.
Bloj, B., & Zilversmit, D. B. (1977) *J. Biol. Chem. 252*, 1613–1619.
Bremer, J., & Greenberg, D. M. (1961) *Biochim. Biophys. Acta 46*, 205–216.
Carter, H. E., Glick, F. J., Norris, W. P., Phillips, G. E., & Harris, R. (1956) *J. Biol. Chem. 170*, 269–283.
Chakrabarti, P., & Khorana, H. G. (1975) *Biochemistry 14*, 5021–5033.
DiCorleto, P. E., Warrach, J. B., & Zilversmit, D. B. (1979) *J. Biol. Chem. 245*, 7795–7802.
Diringer, H., Marggraf, W. D., Koch, M. A., & Anderer, F. A. (1972) *Biochem. Biophys. Res. Commun. 47*, 1345–1352.
Esko, J. D., & Raetz, C. R. H. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5192–5196.
Fleischer, B. (1974) *Methods Enzymol. 31*, 180–191.
Fujino, H., Nakamo, M., Negishi, T., & Ito, S. (1968) *J. Biol. Chem. 243*, 4650–4651.
Helenius, A., McCaslin, D. R., Fries, E., & Tanford, C. (1979) *Methods Enzymol. 56*, 734–749.
Kaller, H. (1961) *Biochem. Z. 334*, 451–456.
Kervina, M., & Fleischer, S. (1974) *Methods Enzymol. 31*, 6–41.
King, T. E. (1967) *Methods Enzymol. 10*, 216–225.
Lee, T. C., Stephens, N., Moehl, A., & Snyder, F. (1973) *Biochim. Biophys. Acta 291*, 86–92.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Lumb, R. H., Kloosterman, A. D., Wirtz, K. W. A., & Van Deenen, L. L. M. (1976) *Eur. J. Biochem. 69*, 15–22.
Marggraf, W. D., Anderer, F. A., & Kanfer, J. N. (1981) *Biochim. Biophys. Acta 664*, 61–73.
Neville, D. M., Jr. (1968) *Biochim. Biophys. Acta 600*, 376–386.
Poorthuis, B. J. H. M., Van Der Krift, T. P., Teerlink, T., Akeroyd, R., Hostetler, K. Y., & Wirtz, K. W. A. (1980) *Biochim. Biophys. Acta 600*, 376–386.
Siakotos, A. N., Kulkarni, S., & Passo, S. (1971) *Lipids 6*, 254–259.
Song, C. S., & Bodansky, O. (1967) *J. Biol. Chem. 242*, 694–699.
Sribney, M., & Kennedy, E. P. (1958) *J. Biol. Chem. 233*, 1315–1322.
Stoffel, W., & Melzner, I. (1980) *Hoppe-Seyler's Z. Physiol. Chem. 361*, 755–771.
Trouet, A. (1974) *Methods Enzymol. 31*, 323–329.
Ullman, M. D., & Radin, N. S. (1974) *J. Biol. Chem. 249*, 1506–1512.
van Golde, L. M. G., Raben, J., Batenburg, J. J., Fleisher, B., Zambrano, F., & Fleischer, S. (1974) *Biochim. Biophys. Acta 360*, 179–192.
Voelker, D. R., & Kennedy, E. P. (1981) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 40*, 1805 (Abstr. No. 1530).
Zakim, D., & Vessey, D. A. (1973) *Methods Biochem. Anal. 21*, 1–38.
Zilversmit, D. B., & Hughes, M. A. (1977) *Biochim. Biophys. Acta 469*, 99–110.
Zwaal, R. F. A., Roelofsen, B., Comfurius, P., & Van Deenen, L. L. M. (1975) *Biochim. Biophys. Acta 406*, 83–96.

Calcium-Independent Stimulation of *Bordetella pertussis* Adenylate Cyclase by Calmodulin†

Donald V. Greenlee, Terrence J. Andreasen, and Daniel R. Storm*

ABSTRACT: *Bordetella pertussis* produces an extracellular adenylate cyclase activity that is present in the culture medium of exponentially growing cells. We have determined that calmodulin (CaM) stimulates this enzyme both in the presence and in the absence of free Ca^{2+}. In the presence of 90 μM Ca^{2+} half-maximal stimulation of the enzyme occurred at 95 pM calmodulin. Comparable levels of calmodulin stimulation were observed when free Ca^{2+} levels were minimized by using EGTA-containing buffers. However, the concentration of CaM required for half-maximal stimulation of *B. pertussis* adenylate cyclase in the presence of 1 nM free Ca^{2+} was 24 nM. The apparent affinity of the enzyme for calmodulin was also significantly enhanced by Mn^{2+}. In addition, troponin I inhibited calmodulin stimulation of the bacterial adenylate cyclase. Photoaffinity cross-linking experiments using azido[^{125}I]calmodulin and *B. pertussis* adenylate cyclase revealed only one major cross-linked product having a molecular weight of 97 000. It is proposed that the catalytic subunit of the calmodulin-sensitive adenylate cyclase is 77 000.

Bordetella pertussis is a small Gram-negative bacilli that is the pathogen responsible for whooping cough (Olson, 1975; Jawetz et al., 1978). The organism adheres to and multiplies on the surface of tracheal and bronchial epithelium, interfering with ciliary function (Linnemann, 1978). Although the molecular basis for *B. pertussis* pathology is undefined, culture media of growing *B. pertussis* contain a number of biologically active components (Morse, 1976). The culture medium of exponentially growing *B. pertussis* accumulates a soluble adenylate cyclase that is insensitive to α-keto acids and guanyl nucleotides (Hewlett et al., 1976; Hewlett & Wolff, 1976). It has been reported that this enzyme is monomeric with a molecular weight of 70 000 on $NaDodSO_4$[1] gels and sucrose

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density gradients (Hewlett & Wolff, 1976). Recently, it was discovered that *B. pertussis* adenylate cyclase is stimulated by calmodulin (CaM), and various criteria for specificity suggested that CaM activation of this enzyme may be functionally significant even though these bacteria do not contain CaM (Wolff et al., 1980). Data reported with whole bacteria indicated that CaM regulation of *B. pertussis* adenylate cyclase differed significantly from CaM stimulation of brain adenylate cyclase. For example, EGTA reversal of CaM stimulation was either minimal or not observed at all.

In the present study, CaM stimulation of adenylate cyclase obtained from *B. pertussis* culture medium was characterized in order to compare this enzyme to other CaM-regulated systems. The sensitivity of this enzyme preparation to CaM in the presence of Ca^{2+} was 500-fold greater than that reported by Wolff et al. (1980) using whole bacteria. Equivalent or higher levels of CaM stimulation were observed even in the presence of 1 nM free Ca^{2+}. In addition, photoaffinity cross-linking studies using azido-[^{125}I]CaM showed only one major CaM binding protein with a molecular weight of 77000.

Materials and Methods

Materials. Bovine serum albumin, parvalbumin, bovine heart cytochrome *c*, sperm whale myoglobin, soybean trypsin inhibitor, and chicken egg ovalbumin were products of Sigma. [^{3}H]cAMP was purchased from New England Nuclear. Troponin I was purified from rabbit muscle by the method of Wilkinson (1974), and calmodulin was purified from bovine brain as previously described (LaPorte et al., 1979).

Adenylate Cyclase Assay. Adenylate cyclase was assayed by the general method of Salmon et al. (1974) except that an ATP-regenerating system was unnecessary. Assay mixtures contained 0.86 mM [α-^{32}P]ATP (100 cpm/pmol), 10 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.5), and 1 mg/mL bovine serum albumin unless otherwise specified. Assays were carried out in triplicate at 30 °C for 10 min, and recovery was monitored with [^{3}H]cAMP. In some assays CaM, $CaCl_2$, $MnCl_2$, or EGTA was present as indicated.

Preparation of Adenylate Cyclase. B. pertussis (strain Tohama phase 1) was grown from a 5% inoculum in supplemented Stainer–Scholte medium (Stainer & Scholte, 1971) for 28 h at 35.5 °C with shaking. This growth medium contained 180 μM added $CaCl_2$. Bacterial suspensions were centrifuged at 5000*g* for 30 min, and the supernatant containing CaM-sensitive adenylate cyclase was rapidly frozen in aliquots and stored at −70 °C. Thawed samples were centrifuged at 150000*g* for 45 min prior to use to ensure removal of particular material. The initial activities of the fresh enzyme in the presence and absence of CaM were 4.4 × 10^6 and 2.4 × 10^4 pmol of cAMP (10 min)$^{-1}$ mg^{-1}, respectively. After 2 months of storage at −70 °C, the basal activity increased to 2.4 × 10^5 units/mg, and activity in the presence of CaM dropped to 2.8 × 10^6 units/mg.

Determination of Ca^{2+} Concentrations. The total calcium concentrations of protein solutions, reagents, and the complete assay cocktail were determined by atomic absorption with a Perkin-Elmer Model 305B atomic absorption spectrophotometer with a graphite furnace. CaM used in assays was extensively dialyzed against 50 mM Tris-HCl, pH 7.5, containing 500 μM EGTA, and the enzyme preparations were desalted into the same buffer to minimize Ca^{2+} concentrations. The total Ca^{2+} concentration of the complete assay cocktail including assay reagents, CaM (2 μM), and the enzyme preparation was 90 μM.

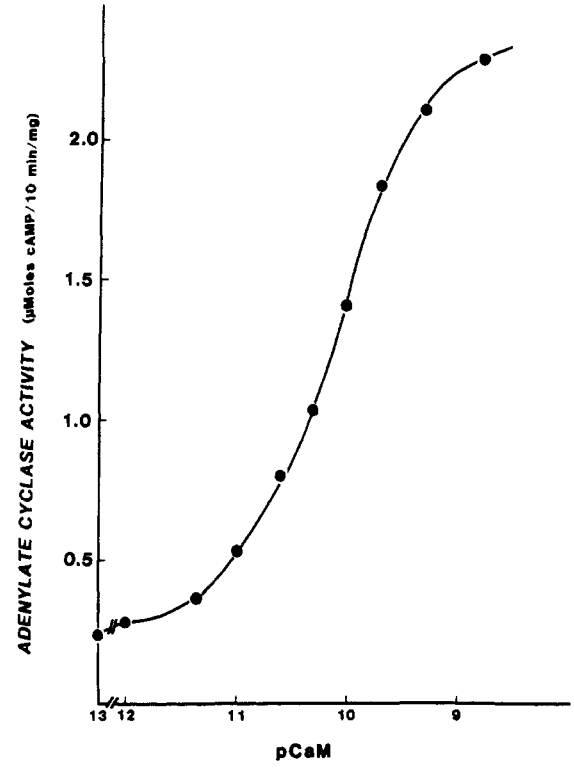

FIGURE 1: Calmodulin stimulation of *B. pertussis* adenylate cyclase in the presence of Ca^{2+}. Adenylate cyclase activity obtained from the culture media of *B. pertussis* was assayed at varying concentrations of CaM in the presence of 90 μM $CaCl_2$. Adenylate cyclase was assayed as described under Materials and Methods. pCaM is −log [calmodulin].

Photoaffinity Labeling with Azido-[^{125}I]CaM. Azido-[^{125}I]CaM was prepared by the method of Andreasen et al. (1981). The *B. pertussis* culture supernatant (50 mL) was lyophilized and resuspended in 10 mL of 50 mM Tris-HCl, pH 7.5, containing 200 μM EGTA (buffer A) and concentrated to 1 mL by ultrafiltration with an Amicon PM-30 membrane. Dilution in buffer A and ultrafiltration concentration were then repeated 3 times. After repeated dilutions and concentrations, the enzyme still retained its sensitivity to CaM. The enzyme solution (100 μL) and azido-[^{125}I]CaM (10 μL) were then incubated in a final volume of 150 μL for 10 min, photolyzed, and run on $NaDodSO_4$ gels and submitted to autoradiography as previously described (Andreasen et al., 1981). The final samples all contained 10 μg of enzyme preparation, 0.33 μM azido-[^{125}I]CaM, and 1.2 mM EGTA in 50 mM Tris-HCl, pH 7.5. When present, underivatized CaM was 7.8 μM.

Results

CaM Stimulation of B. pertussis Adenylate Cyclase. The CaM concentration dependence for stimulation of adenylate cyclase was examined both in the presence and in the absence of free Ca^{2+}. In the presence of 90 μM total Ca^{2+}, half-maximal stimulation occurred at 95 pM CaM (Figure 1). The log interval for 10–90% of maximal stimulation by CaM was 1.9 log units, indicating that CaM stimulation did not show cooperativity. The maximum stimulations by CaM varied considerably from one preparation to another. For example, the data shown in Figure 1, which illustrates a 10-fold stimulation by CaM, were obtained with an enzyme sample that had been frozen and thawed. Preparations of adenylate cyclase

[1] Abbreviations: CaM, calmodulin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; TNI, troponin I; $NaDodSO_4$, sodium dodecyl sulfate; cAMP, adenosine cyclic 3',5'-phosphate; ATP, adenosine 5'-triphosphate; Tris, tris(hydroxymethyl)aminomethane.

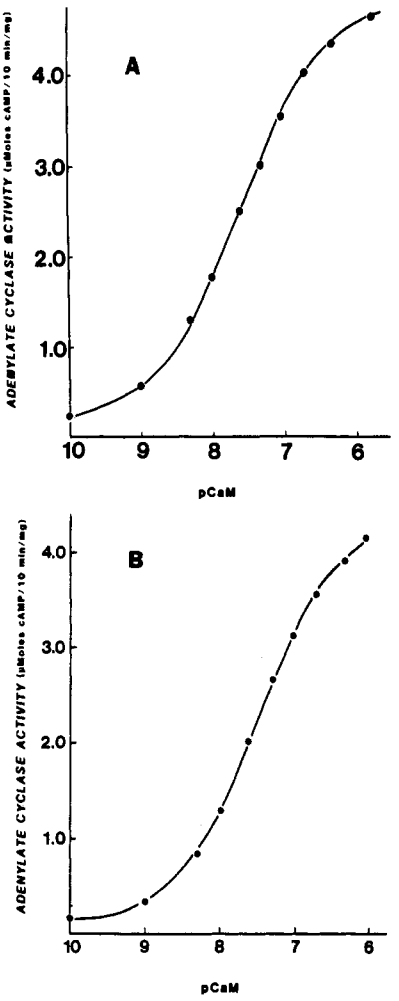

FIGURE 2: Calmodulin activation of *B. pertussis* adenylate cyclase in the absence of free Ca^{2+}. (A) *B. pertussis* culture media containing adenylate cyclase was desalted into 50 mM Tris-HCl, pH 7.5, containing 500 μM EGTA with a Bio-Rad P2 column equilibrated in this buffer. Adenylate cyclase activity was assayed as a function of calmodulin concentration as described under Materials and Methods. pCaM is -log [calmodulin]. The concentration of EGTA in the assay mixture was 200 μM, and total Ca^{2+} was 90 μM. (B) Same as (A) except the concentration of EGTA in the assay mixture was 5 mM. Free Ca^{2+} concentration was ≤1 nM.

assayed directly from the growth media without freezing were stimulated approximately 200-fold by CaM.

When free Ca^{2+} was minimized by desalting of the enzyme into an EGTA-containing buffer and the enzyme was assayed in the presence of 200 μM EGTA, CaM stimulated the enzyme 23-fold (Figure 2A). However, the concentration of CaM required for half-maximal stimulation of adenylate cyclase in the absence of free Ca^{2+} was 24 nM. In order to be absolutely certain that there was insufficient free Ca^{2+} to account for the observed stimulation by CaM, we carried out an analogous experiment in the presence of 5 mM EGTA (Figure 2B). Again, half-maximal stimulation occurred at 24 nM CaM. The total Ca^{2+} concentration in the complete assay cocktail containing all reagents, CaM, and the enzyme preparation was 90 ± 10 μM. In the presence of 5 mM EGTA and 10 mM $MgCl_2$ at pH 7.5 it can be calculated that free Ca^{2+} is no greater than 1 nM (Schmid & Reilley, 1957; Schwarzenbach, 1957; Sillen & Martell, 1964). Since the dissociation constants for CaM·Ca^{2+} complexes are 1 μM or greater even in the presence of CaM binding proteins (Keller et al., 1981), the CaM stimulation reported in Figure 2B must be due to CaM and not to CaM·Ca^{2+}_n. The data reported in Figures 1 and 2 were obtained with the same enzyme preparation, and it is evident that CaM stimulation is approximately 2-fold greater in the absence of free Ca^{2+} than in the presence of Ca^{2+}. However, the Ca^{2+} concentration used for the experiment in Figure 1 was not necessarily optimal for CaM stimulation, and the differences in maximal activity seen in the absence and presence of Ca^{2+} may be due in part to Ca^{2+} inhibition at higher Ca^{2+} concentrations (see Figure 5).

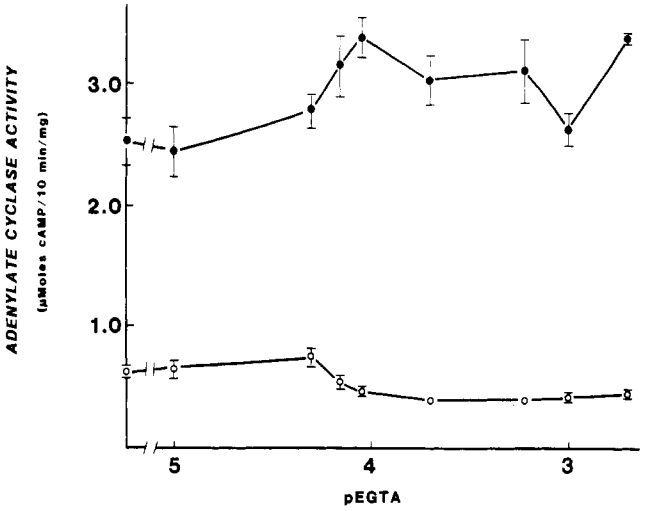

FIGURE 3: Effect of EGTA on CaM stimulation of *B. pertussis* adenylate cyclase. Growth media containing adenylate cyclase was assayed with increasing concentrations of EGTA in the presence (●) or absence (○) of 100 nM CaM. The total concentration of Ca^{2+} in the complete assay mixture was 90 μM. pEGTA is -log [EGTA].

Effects of EGTA on CaM Stimulation. The data reported above indicated that CaM stimulated *B. pertussis* adenylate cyclase in the absence of significant levels of free Ca^{2+} (≤1 nM Ca^{2+}). This general observation was confirmed by examining CaM stimulation as a function of increasing EGTA up to 3 mM EGTA. As shown in Figure 3, EGTA at concentrations as high as 3 mM did not reverse CaM stimulation of the enzyme. These data are consistent with that reported in Figure 2B where it was shown that 5 mM EGTA did not inhibit CaM stimulation when CaM was 100 nM.

Kinetics for Loss of CaM Sensitivity. CaM stimulation of *B. pertussis* adenylate cyclase was quite variable depending upon storage conditions. Therefore, the enzyme preparation was incubated at 4 °C for various periods of time up to 25 h and then assayed for basal and CaM-stimulated activities (Figure 4). Initially, this preparation was stimulated 8-fold by CaM. After 25 h, CaM stimulation was lost. During this time period the basal activity increased 3.5-fold. This behavior is similar to results obtained with other CaM-regulated enzymes in which limited proteolysis has been shown to activate the enzymes with loss of CaM sensitivity (Klee et al., 1980). The addition of PMSF did not significantly affect the rate for loss of CaM stimulation. The apparent stimulation by PMSF was due to low amounts of ethanol used as a solvent for PMSF. Ethanol (1%) by itself activated the enzyme approximately 30%. Incubation with 250 mM sucrose also did not significantly affect the rate of increase in basal activity or loss in CaM stimulation. Although the basis for these activity changes was not examined in any further detail, it seems likely, by analogy with other CaM-regulated enzymes, that they may be due to limited proteolysis.

Ca^{2+} and Mn^{2+} Stimulation of B. pertussis Adenylate Cyclase. Adenylate cyclase activity was assayed as a function of Ca^{2+} concentration in the presence of no added CaM, 0.1 nM CaM, and 4.8 μM CaM (Figure 5). In all cases, 200 μM EGTA was present and the Ca^{2+} concentrations given in

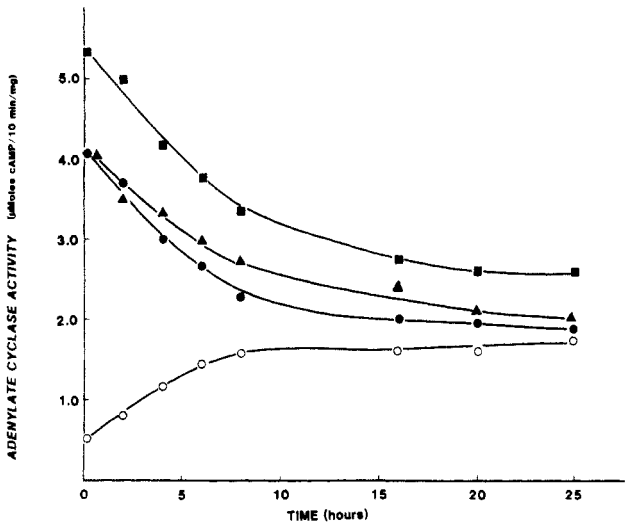

FIGURE 4: Kinetics for loss in CaM sensitivity of *B. pertussis* adenylate cyclase. Adenylate cyclase in the growth media was incubated for various periods of time at 4 °C with no additions (○, ●), in the presence of 1 mM PMSF (■), or in the presence of 250 mM sucrose (▲). The enzyme was then assayed in either the presence (●, ■, ▲) or absence (○) of 5 μM CaM.

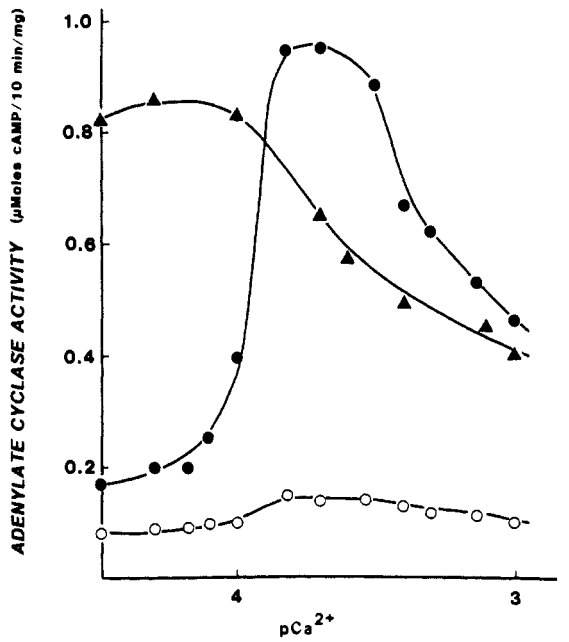

FIGURE 5: Ca^{2+} dependency for calmodulin activation of *B. pertussis* adenylate cyclase. Cyclase from *B. pertussis* culture media was desalted into 50 mM Tris-HCl, pH 7.5, containing 500 μM EGTA and assayed as a function of varying Ca^{2+} concentrations. (○) Without added CaM; (●) in the presence of 0.1 nM calmodulin; (▲) in the presence of 4.8 μM calmodulin. EGTA concentration in the assay was 200 μM. pCa^{2+} is −log $[Ca^{2+}]$.

Figure 4 are total $CaCl_2$ added. In the absence of CaM, there was little or no effect of Ca^{2+} on basal enzyme activity. In the presence of 4.8 μM CaM, which would saturate the enzyme even in the absence of free Ca^{2+} (Figure 2), added $CaCl_2$ in excess of EGTA simply inhibited the enzyme. At 0.1 nM CaM, which corresponds to the concentration of CaM for half-maximal stimulation in the presence of Ca^{2+} (Figure 1), Ca^{2+} stimulated the enzyme at low concentrations and progressively inhibited adenylate cyclase at higher concentrations. The biphasic response to Ca^{2+} in the presence of CaM is similar to that exhibited by the CaM-sensitive adenylate cyclase from bovine cerebral cortex (Westcott et al., 1979). Maximal CaM stimulation occurred at approximately 0.05

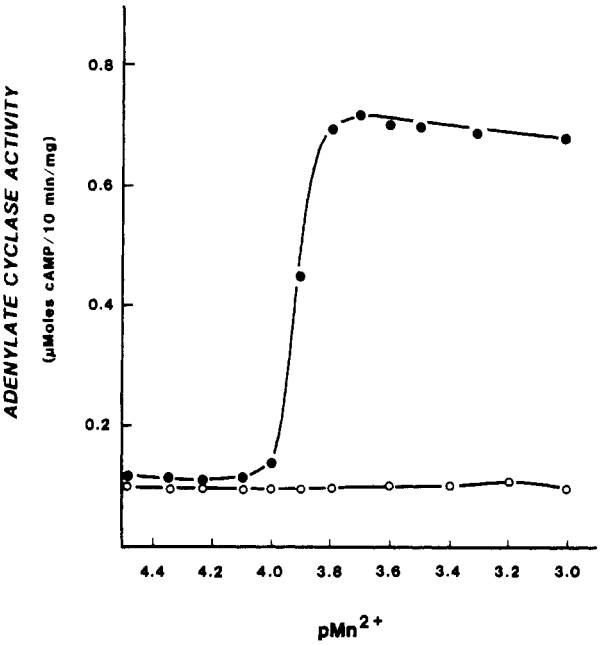

FIGURE 6: Mn^{2+} dependency for CaM stimulation of *B. pertussis* adenylate cyclase. Adenylate cyclase was desalted into 50 mM Tris-HCl (pH 7.5) containing 500 μM EGTA and then assayed for adenylate cyclase activity in the absence (○) or presence (●) of 0.1 nM CaM. The final EGTA concentration in the assays was 200 μM. pMn^{2+} is −log $[Mn^{2+}]$.

μM free Ca^{2+}, which is similar to that reported by Piascik et al. (1980) for brain adenylate cyclase. These data are consistent with the results presented in Figures 1 and 2, which illustrated that CaM can stimulate the enzyme even in the absence of free Ca^{2+}. High levels of EGTA did not inhibit CaM stimulation of the enzyme when CaM concentrations were greater than 1 μM since the apparent dissociation constant for the CaM·adenylate cyclase complex was 24 nM in the absence of Ca^{2+}.

Mn^{2+} binds to CaM (Wolff et al., 1977) and will support CaM stimulation of the CaM-sensitive adenylate cyclase from bovine brain (Keller et al., 1980). Therefore, the activity of *B. pertussis* adenylate cyclase was examined as a function of Mn^{2+} concentration in the absence and presence of 0.1 nM CaM (Figure 6). All assays were carried out in the presence of 10 mM $MgCl_2$. Basal adenylate cyclase activity was unaffected by $MnCl_2$ at concentrations up to 1 mM. In contrast to most other adenylate cyclase activities, the *B. pertussis* enzyme actually exhibits higher activity with $MgCl_2$ than with $MnCl_2$. Mn^{2+} did, however, support CaM stimulation of the enzyme with maximal activities comparable to that observed with Ca^{2+}. In contrast to Ca^{2+}, higher levels of Mn^{2+} did not inhibit the enzyme in the presence or absence of CaM. These observations suggest that the Ca^{2+} inhibition reported in Figure 4 is not mediated through CaM.

Inhibition of CaM Stimulation by TNI. TNI forms a 1:1 complex with CaM (LaPorte et al., 1980; Andreasen et al., 1981), and its affinity for CaM is enhanced approximately 3000-fold in the presence of Ca^{2+} (Keller et al., 1981). In addition, TNI inhibited CaM stimulation of the Ca^{2+}-sensitive phosphodiesterase and other CaM-stimulated enzymes (LaPorte et al., 1980), suggesting that TNI binds to the same domain on CaM as other CaM-regulated enzymes. Therefore, the influence of TNI on CaM stimulation of *B. pertussis* adenylate cyclase was examined (Figure 7). TNI caused a slight stimulation of basal adenylate cyclase activity, which was probably due to contamination by low levels of CaM. When TNI and CaM were preincubated prior to addition of

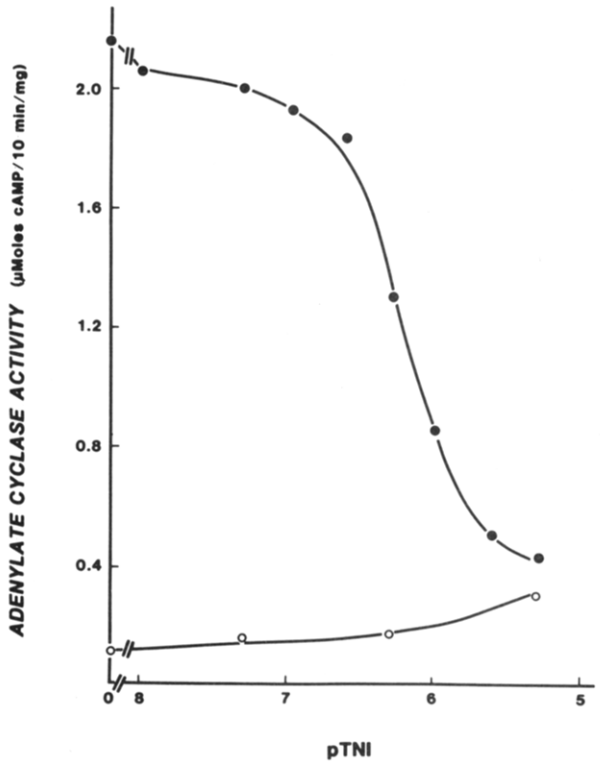

FIGURE 7: Inhibition of CaM stimulation of *B. pertussis* adenylate cyclase by troponin I (TNI). Varying concentrations of TNI were preincubated with 1 nM CaM and 100 μM $CaCl_2$ for 1 h, and then adenylate cyclase was assayed in either the absence (○) or presence (●) of the TNI·CaM complex as described under Materials and Methods. pTNI is –log [TNI].

adenylate cyclase, CaM stimulation was inhibited by TNI. Half-maximal inhibition of CaM stimulation occurred at approximately 0.9 μM TNI. These data indicate that the Ca^{2+}_4·CaM·TNI complex does not stimulate *B. pertussis* adenylate cyclase, which is analogous behavior to that of other CaM-regulated enzymes.

Photoaffinity Labeling with Azido-[^{125}I]CaM. We recently reported that azido-[^{125}I]CaM retains its affinity for CaM binding proteins and demonstrated that this reagent can be used to specifically photoaffinity label CaM binding subunits of TNI, myosin light chain kinase, the Ca^{2+}-sensitive phosphodiesterase, and the Ca^{2+},Mg^{2+}-ATPase (Andreasen et al., 1981). Samples of the impure *B. pertussis* adenylate cyclase were photoaffinity labeled with azido-[^{125}I]CaM in an attempt to identify the enzyme on $NaDodSO_4$ gels. Since data discussed previously indicated that CaM interacts with this enzyme even in the absence of free Ca^{2+}, photoaffinity labeling was carried out in the presence of 1.2 mM EGTA with no added Ca^{2+}. The total calcium in the reaction mixture including all reagents was 10 μM. In the presence of 1.2 mM EGTA at pH 7.5 the concentration of free calcium was approximately 0.3 nM (Sillen & Martell, 1964). The concentration of azido-[^{125}I]CaM was 0.33 μM, which would be sufficient to saturate adenylate cyclase under these conditions. The apparent activation constant was 24 nM in the presence of 1 nM free Ca^{2+} (Figure 2). Photoaffinity labeling experiments revealed one major cross-linked polypeptide with a molecular weight of 97 000 on $NaDodSO_4$ gels (Figure 8). This cross-linked product was not produced when photoaffinity labeling was carried out in the presence of excess unmodified CaM. There were also minor bands at 60 000 and 130 000 daltons. In all cases examined thus far, the stoichiometry for binding of CaM to CaM binding subunits is 1:1 (Andreasen et al., 1981). In addition, the apparent molecular weight of

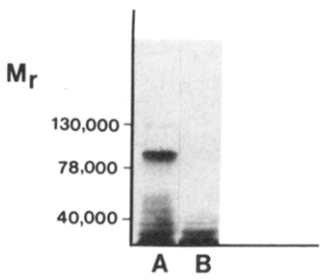

FIGURE 8: Photoaffinity labeling of *B. pertussis* adenylate cyclase with azido-[^{125}I]CaM. Adenylate cyclase in culture supernatant was labeled with azido-[^{125}I]CaM, run on $NaDodSO_4$ gels, and autoradiographed as described under Materials and Methods. (A) 10 μg of enzyme preparation, 0.33 μM azido-[^{125}I]CaM, and 1.2 mM EGTA in 50 mM Tris-HCl, pH 7.5; (B) same as (A) except that 7.8 μM underivatized CaM was present.

CaM on $NaDodSO_4$ gels was 20 000 on the gel system used in this study. Therefore, the major CaM binding polypeptide showing significant affinity for CaM in the absence of Ca^{2+} has a molecular weight of approximately 77 000. When photoaffinity labeling was carried out in the presence of Ca^{2+}, there were other cross-linked products produced in addition to the three seen in the absence of Ca^{2+}. The molecular weights of these cross-linked polypeptides were 85K, 103K, and 115K.

Discussion

The existence of an extracellular adenylate cyclase that is stimulated as much as 200-fold by CaM is quite interesting, particularly since *B. pertussis* does not contain calmodulin (Wolff et al., 1980). The physiological function of this enzyme is not known. However, it is quite possible that *B. pertussis* adenylate cyclase has evolved in response to host calmodulin and plays an important role for the host–parasite relationship. Alternatively, *B. pertussis* may contain a CaM-like regulator. However, there is currently no evidence for such an activity in *B. pertussis*, and the sensitivity of the enzyme to exogenous CaM in crude extracts strongly argues against the existence of such a factor.

This study was initiated because the bacterial adenylate cyclase apparently does not contain the guanyl nucleotide regulatory component (G/F) nor is it regulated by guanyl nucleotides, hormones, or fluoride ion. Previous studies with brain adenylate cyclase suggested that G/F may be required for CaM regulation of that enzyme (Toscano et al., 1979). It is now clear that G/F is not an obligatory requirement for CaM stimulation of adenylate cyclase activities. It was felt that a comparison between CaM regulation of the bacteria enzymes and that of brain enzymes may provide insight for the mechanism for CaM stimulation.

In the presence of Ca^{2+}, the activation constant for CaM stimulation of the culture media adenylate cyclase was 95 pM. In contrast, Wolff et al. (1980) reported that half-maximal stimulation of the enzyme in intact microorganisms occurred at 45 nM, a difference of approximately 500-fold. This significant quantitative difference between the two studies is most likely due to the differences in the enzyme preparations used. Regardless, the apparent affinity of *B. pertussis* adenylate cyclase for CaM is quite high even in the absence of Ca^{2+}, suggesting that the interaction may be specific and functionally significant. If indeed the interaction between extracellular adenylate cyclase and CaM is physiologically significant, it seems unlikely that fluctuations in free Ca^{2+} would play a significant role for this process.

In contrast to the CaM-sensitive cyclic nucleotide phosphodiesterase, *B. pertussis* adenylate cyclase is stimulated by

CaM even in the absence of significant levels of free Ca^{2+}. These experiments were carried out by desalting the enzyme into an EGTA-containing buuffer and carrying out assays in the presence of 5 mM EGTA with Ca^{2+}-free CaM. From the known dissociation constant of EGTA·Ca^{2+} (Sillen & Martell, 1964) it is estimated that in the presence of 5 mM EGTA, 10 mM Mg^{2+}, and 90 μM total Ca^{2+} that the concentration of free Ca^{2+} is 1 nM or less at pH 7.5. Since the free Ca^{2+} concentration was 3 orders of magnitude lower than the dissociation constant for CaM or CaM complexed to CaM binding proteins (Keller et al., 1981), the observed stimulations could not be due to CaM·Ca^{2+}_n. It was also determined that *B. pertussis* adenylate cyclase is absorbed to CaM–Sepharose in the presence of 5 mM EGTA (data not shown). Although the enzyme could be eluted from the column with free CaM, recovery of activity was low because of the slow exchange rate between free CaM and immobilized CaM. Under identical conditions, the phosphodiesterase was not stimulated by CaM, even at CaM concentrations as high as 50 μM (unpublished observations). To our knowledge, this is the first data indicating Ca^{2+}-independent stimulation of any enzyme by CaM. The apparent affinity of the enzyme for CaM was, however, increased approximately 200-fold by Ca^{2+}. It was originally predicted by Keller et al. (1980) that CaM should interact with CaM binding proteins in the absence of Ca^{2+}. Other characteristics of CaM stimulation of *B. pertussis* adenylate cyclase are similar to other CaM-regulated systems. For example, Mn^{2+} substituted for Ca^{2+} in supporting CaM stimulation. TNI inhibited CaM stimulation, suggesting that the CaM binding domain that interfaces with TNI is the same as that which interacts with the bacterial adenylate cyclase.

Hewlett & Wolff (1976) reported that the molecular weight of a purified adenylate cyclase from *B. pertussis* is 70 000. It was subsequently reported that the purified enzyme is not stimulated by CaM plus Ca^{2+}. This CaM insensitivity was attributed to a loss of another factor needed by the system (Wolff et al., 1980). An alternative interpretation is suggested by data reported in this study. It is quite possible that CaM stimulation is lost when the catalytic subunit is partially proteolyzed. Cross-linking studies with the CaM-sensitive enzyme revealed several CaM binding polypeptides. The major polypeptide exhibiting affinity for CaM in the absence of Ca^{2+} has a molecular weight of 77 000, slightly larger than that reported for the purified, CaM-insensitive enzyme. We suspect that this polypeptide is the catalytic subunit of the enzyme and that proteolysis leads to a smaller 70 000-dalton subunit that no longer interacts with CaM. Quite clearly, our data do not allow for the unambiguous identification of the adenylate cyclase CaM binding subunit, and this question can only be resolved by studies using more highly purified enzyme.

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References

Andreasen, T. J., Keller, C. H., LaPorte, D. C., Edelman, A. M., & Storm, D. R. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2782.

Hewlett, E., & Wolff, J. (1976) *J. Bacteriol. 127*, 890.

Hewlett, E. L., Urban, M. A., Manclark, C. R., & Wolff, J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1926.

Jawetz, E., Melnick, J. L., & Adlberg, E. A. (1978) *Review of Medical Microbiology*, 13th ed., Lange Medical Publications, Los Altos, CA.

Keller, C. H., LaPorte, D. C., Toscano, W. A., Storm, K. R., & Westcott, K. R. (1980) *Ann. N.Y. Acad. Sci. 356*, 205.

Keller, C. H., Olwin, B. B., LaPorte, D. C., & Storm, D. R. (1981) *Biochemistry 20*, 3965.

Klee, C. B., Crouch, T. H., & Richman, P. G. (1980) *Annu. Rev. Biochem. 49*, 489.

LaPorte, D. C., Toscano, W. A., & Storm, D. R. (1979) *Biochemistry 18*, 2820.

LaPorte, D. C., Maso, B. M., & Storm, D. R. (1980) *Biochemistry 19*, 3814.

Linnemann, C. C., Jr. (1978) *DHEW Publ. (NIH) (U.S.) NIH* 79-1830, 3.

Morse, S. L. (1976) *Adv. Appl. Microbiol. 20*, 9.

Olson, L. C. (1975) *Medicine (Baltimore) 54*, 427.

Piascik, M. T., Wisler, P. L., Johnson, C. L., & Potter, J. D. (1980) *J. Biol. Chem. 255*, 4176.

Salmon, Y., Londos, C., & Rodbell, M. (1974) *Anal. Biochem. 58*, 541.

Schmid, R. W., & Reilley, C. N. (1957) *Anal. Chem. 29*, 264.

Schwarzenbach, G. (1957) *Complexometric Titrations*, Interscience, New York.

Sillen, L. G., & Martell, A. E. (1964) *Stability Constants of Metal Ion Complexes*, 2nd ed., Special Publication no. 17, The Chemical Society, Burlington House, London, England.

Stainer, D. W., & Scholte, M. J. (1971) *J. Gen. Microbiol. 63*, 211.

Toscano, W. A., Westcott, K. R., LaPorte, D. C., & Storm, D. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5582.

Westcott, K. P., LaPorte, D. C., & Storm, D. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 204.

Wilkinson, J. M. (1974) *Biochim. Biophys. Acta 359*, 379.

Wolff, D. J., Poririer, P. G., Brostrom, C. O., & Brostrom, M. A. (1977) *J. Biol. Chem. 252*, 4108.

Wolff, J., Cook, G. H., Goldhammer, A. R., & Berkowitz, S. A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3841.