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Alteration of Intracellular cAMP Levels and Beating Rates of Cultured Chick Cardiac Cells by *Bordetella pertussis* Adenylate Cyclase

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SUMMARY

Bordetella pertussis, the pathogen responsible for whooping cough, releases a soluble calmodulin-sensitive adenylate cyclase into its culture medium which enters several different types of animal cells and elevates intracellular cAMP. In this study, the influence of *B. pertussis* adenylate cyclase on intracellular cAMP levels of cultured chick cardiac cells and the beating rates of chick cardiac cell aggregates was examined. Treatment with *B. pertussis* adenylate cyclase caused up to a 60-fold increase in intracellular cAMP which was significantly greater than that caused by forskolin or isoproterenol. Increases in intracellular cAMP caused by *B. pertussis* adenylate cyclase were observed within 2 min after treating cells with the enzyme, and binding of calmodulin to the enzyme inhibited these effects. In addition, high concentrations of the enzyme completely inhibited the beating of cardiac cells. However, lower concentrations of the adenylate cyclase accelerated beating rates 30–40% and cardiac cells continued to beat at an accelerated rate for at least 30 min. These data indicate that *B. pertussis* adenylate cyclase invades chick cardiac cells and catalyzes significant increases in intracellular cAMP. It is proposed that the effect of the enzyme on the beating rates of heart cell aggregates may be due to alteration of intracellular cAMP levels.

The culture medium of growing *Bordetella pertussis* contains a number of biologically active components which may play a role in the pathogenesis of whooping cough (1, 2). One of these, IAP, has been purified from the culture medium of the bacteria (3–5). IAP attenuates receptor-mediated inhibition of adenylate cyclase in a variety of mammalian cell types by catalyzing the ADP-ribosylation of the inhibitory guanyl nucleotide component of the adenylate cyclase system (6–8). The attenuation of receptor-mediated inhibition can result in an increase in intracellular cAMP levels.

Another factor, which may play a role in the pathogenesis of whooping cough, is the adenylate cyclase from *B. pertussis*. Adenylate cyclase activity was originally detected in the culture medium of *B. pertussis* (9, 10). It was subsequently discovered that this enzyme is stimulated by CaM even though *B. pertussis* does not contain CaM (11). Weiss *et al.* (12, 13) have determined that *B. pertussis* mutants deficient in adenylate cyclase are avirulent. Recently, the *B. pertussis* adenylate cyclase has been extensively purified by Shattuck *et al.* (14) to a specific activity of 600 μmol of cAMP/min/mg. Confer and Eaton (15), and Hanski and Farfel (16) have shown that incubation of crude preparations of the *B. pertussis* adenylate cyclase with several types of animal cells incuding lymphocytes, S49 lymphoma cells, and neutrophils caused increases in intracellular cAMP. Shattuck and Storm (17) demonstrated that a more highly purified *B. pertussis* adenylate cyclase, lacking IAP, catalyzed the formation of cAMP from intracellular ATP in human erythrocytes and neuroblastoma cells.

In this study, we have examined the influence of *B. pertussis* adenylate cyclase on intracellular cAMP levels of cultured chick cardiac cells and the beating rate of cell aggregates. Invasion of cardiac cells by *B. pertussis* adenylate cyclase was of interest since the beating rate of cardiac cells is sensitive to intracellular cAMP and could serve as a useful physiological assay for entry of the enzyme into animal cells. The data reported in this study indicate that cardiac cells are rapidly invaded by *B. pertussis* adenylate cyclase with measurable effects on the beating rates of cardiac cells.

Materials and Methods

QAE-Sephadex and cyanogen bromide-activated Sepharose 4B were purchased from Pharmacia. Fetuin, ATP, cAMP, theophylline, and protein kinase were from Sigma. [^{32}P]NAD and α-[^{32}P]ATP were purchased from New England Nuclear, and [^{3}H]cAMP was purchased from International Chemical Nuclear. High and low molecular weight standards for SDS-gel electrophoresis were from BioRad. IAP was supplied by List Biologics. Medium M199 and fetal bovine serum were purchased from GIBCO. All other reagents were of the finest available grade from commercial sources.

ABBREVIATIONS: IAP, islet-inactivating protein; CaM, calmodulin; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetate.

Adenylate cyclase assay. Adenylate cyclase was assayed at 30° by the general method of Salomon *et al.* (18), using α-[^{32}P]ATP as a substrate and [^{3}H]cAMP to monitor product recovery. In a final volume of 250 μl, each assay contained 20 mM Tris-Cl, pH 7.5, 1 mM α-[^{32}P] ATP (10 cpm/pmol), 5 mM $MgCl_2$, and 0.1% bovine serum albumin. CaM (2.4 μM) was included in some assays as indicated. All results are presented as the mean of triplicate assays with standard errors of less than 5%. One unit of adenylate cyclase activity is defined as that activity which will catalyze the formation of 1 μmol of cAMP/min under standard assay conditions. Protein concentrations were determined by the method of Peterson (19) or a modification of the method of Lowry *et al.* (20).

Intracellular cAMP determination. Cultured chick heart cells on tissue culture plates were incubated at 37° for various periods of time with medium M199 with 5 mM theophylline or adenylate cyclase preparations in M199 with theophylline. After incubation, the medium was removed and the cells were washed with ice-cold PBS before assaying for cAMP. Intracellular cAMP was assayed by the method of Gilman (21) as modified by Martin *et al.* (22) using purified cAMP-dependent protein kinase.

Preparation of CaM. CaM was prepared from bovine brain by the procedure of Dedman *et al.* (23) as modified by Olwin *et al.* (24).

Fetuin Sepharose. The fetuin affinity resin was prepared by coupling 200 mg of fetuin with 25 g of cyanogen bromide-activated Sepharose 4B according to the manufacturer's recommended procedure.

Partial purification of the CaM-sensitive adenylate cyclase from *B. pertussis*. *B. pertussis* adenylate cyclase was purified by the method of Shattuck *et al.* (14) and a brief description of that purification is presented here. *B. pertussis* (Tohama phase 1) was grown from a 5% inoculum in supplemented Stainer-Scholte medium (25) at 35.5° with shaking until A_{650} = 0.5. Bacterial suspensions were centrifuged in a Beckman J6-3B for 1 hr at 48,000 rpm and the culture medium containing adenylate cyclase activity was removed. QAE-Sephadex (500 ml) equilibrated in 20 mM Tris-HCl (pH 7.5), 20 mM NaCl, and 2 mM $MgCl_2$ was added to 10 liters of *B. pertussis* culture medium and stirred for 1 hr. The resin was poured into a column, and the adenylate cyclase activity was eluted in two distinct peaks of activity with a linear 40 mM to 1 M NaCl gradient.

Peak I adenylate cyclase activity was applied to a 10-ml fetuin-Sepharose column. The protein that flowed through the fetuin-Sepharose column was concentrated 10-fold by Amicon ultrafiltration using a PM-10 membrane. This enzyme preparation had a specific activity of 100 μmol of cAMP/min/mg. All steps of the purification were carried out at 4°.

Detection of contaminating IAP. The presence of IAP in enzyme preparations was detected by its ability to catalyze the ADP-ribosylation of GTP-binding proteins in cerebral cortex membranes (26, 27). Samples of IAP, used as a standard, and adenylate cyclase fractions which had been run through a fetuin-Sepharose column were incubated with 20 mM dithiothreitol for 30 min at 30°. Bovine brain membranes prepared by the method of Andreasen *et al.* (28) were then incubated for 1 hr at 30° with the IAP sample in a buffer containing 10 mM thymidine, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM ATP, 10 μM NAD, 40 mM Tris-Cl (pH 8), and 20 μCi of [^{32}P]NAD. The reaction was stopped by the addition of 1 ml of cold 100 mM Tris-Cl (pH 8). Membranes were washed and applied to a 10% polyacrylamide slab gel in SDS and autoradiographed to detect labeling of IAP substrates. There was no detectable IAP activity in the adenylate cyclase preparation used for cell invasion studies.

SDS-gel electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out as described by Laemmli (29) and stained with Coomassie Brilliant Blue.

Cick cardiac monolayer cell cultures. Cardiac cell cultures were prepared from 9-day-old chick embryos as described by Hunter and Nathanson (30). The cells were grown in an atmosphere of 5% CO_2/95% humidified air. The medium was changed on day 3 in culture and experiments were conducted on day 4. The cells were grown to 80–100% confluency in collagen-coated culture dishes prior to the start of each experiment.

Chick cardiac aggregate cell cultures. Cardiac cell cultures were prepared as described above with the following modifications. The cells were placed in polypropylene tubes and grown in cell culture medium in a 95% air/5% CO_2 incubator at 37° for 3 days. Chick cardiac cells do not adhere to polypropylene and thus form aggregates. The cells were then triturated with a fire-polished Pasteur pipette and plated onto collagen-coated 35-mm dishes to which the resultant cell aggregates attached. Cultures were used for beating rate measurements 1–2 days later.

Beating rate determination. The beating rate of cardiac aggregate cell cultures was determined by measuring the time required for 20 beats using an electric stopwatch. Control beating rates were 100 ± 5 beats/min. The beating rates of control cell aggregates remained constant for 60 min or longer. All results are presented as the mean of triplicate determinations with standard errors of less than 5%. Plates were kept in a CO_2 incubator and only removed for brief periods (<1 min) to determine beating rates.

Results

Entry of *B. pertussis* adenylate cyclase into cultured chick cardiac cells. Cultured chick cardiac cells were incubated at 37° for 15 min with partially purified *B. pertussis* adenylate cyclase (1.2 units/plate) which had been passed over a fetuin-Sepharose column to remove contaminating IAP. In addition, cardiac cells were also treated with 10 μM isoproterenol, 100 μM forskolin, or a combination of these two drugs in order to compare the effect of the bacterial enzyme on intracellular cAMP levels with that caused by activators of endogeneous adenylate cyclase. All incubations were carried out in media containing 5 mM theophylline to suppress phosphodiesterase activity. After 20 min of incubation at 37°, the cells were washed, lysed, and assayed for intracellular cAMP (Fig. 1). *B. pertussis* adenylate cyclase caused a 15-fold increase in intracellular cAMP (3700 pmol of cAMP/mg of protein) relative to controls treated only with theophylline-containing media. The levels of intracellular cAMP obtained by treatment with the bacterial enzyme were significantly higher than those stimulated by isoproterenol or forskolin alone. The maximum level of intracellular cAMP obtainable by stimulation of the endogeneous adenylate cyclase with a combination of forskolin and isoproterenol was 5600 pmol of cAMP/mg. However, treatment of cardiac cells with higher concentrations of the bacterial enzyme, 3.0 units/plate, gave intracellular cAMP levels up to 13,000 pmol of cAMP/mg (Fig. 2), illustrating that cAMP production catalyzed by the internalized bacterial adenylate cyclase can exceed the cAMP synthetic capacity of the cell. These data suggest that *B. pertussis* adenylate cyclase enters chick cardiac cells and catalyzes the synthesis of cAMP from intracellular ATP.

The increase in intracellular cAMP catalyzed by *B. pertussis* adenylate cyclase were inhibited approximately 80% by binding of CaM to the enzyme, even though CaM stimulated the bacterial adenylate cyclase activity 5-fold. These data suggest that the adenylate cyclase · CaM complex cannot enter cardiac cells, and rule out the possibility that increases in intracellular cAMP were due to synthesis of cAMP extracellularly by the bacterial enzyme and transport of cAMP inside the cell. If the latter were true, it would be difficult to rationalize inhibition of intracellular cAMP increases by CaM, which stimulates the enzyme. Furthermore, there is no obvious source for extracellular ATP since the cells were healthy and intact during the

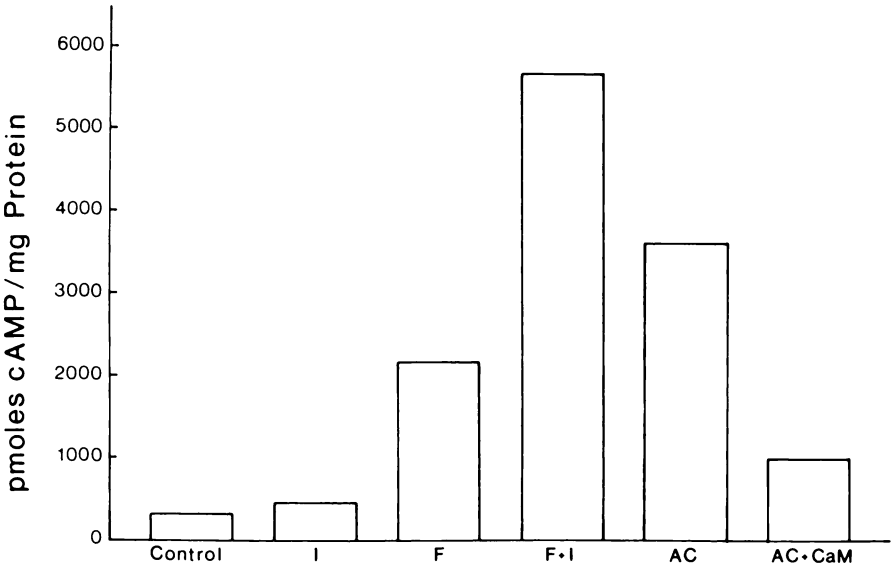

Fig. 1. Increase in intracellular cAMP levels in cultured chick cardiac cells stimulated by *B. pertussis* adenylate cyclase. Chick cardiac cells were grown to 80–100% confluency as described in Materials and Methods. Cultures were washed with serum-free M199, then preincubated with 5 mM theophylline in serum-free M199 for 20 min at 37°. After preincubation, cells were incubated with M199 + 5 mM theophylline which contained: no addition (*Control*), 10 μM isoproterenol (*I*), 100 μM forskolin (*F*), 100 μM forskolin + 10 μM isoproterenol (*F + I*), 1.2 units/plate *B. pertussis* adenylate cyclase (*AC*), or 1.2 units/plate *B. pertussis* adenylate cyclase with 5.4 μM CaM (*AC + CaM*), for 20 min at 37°. Cells were then washed with ice-cold PBS and then assayed for intracellular cAMP.

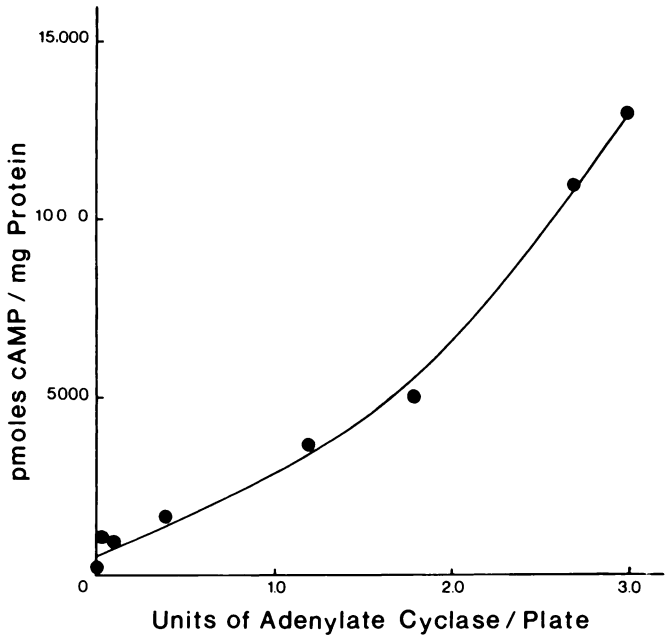

Fig. 2. Dose response curve for increases in intracellular cAMP caused by *B. pertussis* adenylate cyclase. Chick cardiac cells were grown to 80–100% confluency as described in Materials and Methods. Cultures were washed with serum-free M199, then preincubated with 5 mM theophylline in serum-free M199 for 20 min at 37°. After preincubation, cells were incubated with M199 + 5 mM theophylline which contained the indicated adenylate cyclase activity (units = μmol of cAMP produced/min) for 20 min at 37°. Cells were then washed with ice-cold PBS and then assayed for intracellular cAMP.

course of the assay. This same preparation of *B. pertussis* adenylate cyclase catalyzed the production of intracellular cAMP when it was incubated with human erythrocytes (17). Since human erythrocytes showed no endogeneous adenylate cyclase activity, it seems highly likely that this enzyme invades several types of animal cells including chick heart cells and human erythrocytes.

The experiments described above were all carried out in the presence of theophylline to allow maximum cAMP production. Similar experiments were also carried out in the absence of theophylline to determine whether *B. pertussis* adenylate cyclase can elevate intracellular cAMP without inhibition of phosphodiesterase activity. Under these conditions, the bacterial enzyme (1.2 units/ml) elevated cAMP levels to 1800 pmol of cAMP/mg, which is approximately 50% lower than values obtained with theophylline present. These data illustrate that the rate of cAMP production catalyzed by internalized *B. pertussis* adenylate cyclase can exceed the rate of cAMP hydrolysis catalyzed by endogeneous phosphodiesterase activity.

Dose response curves for *B. pertussis* adenylate cyclase. The enzyme concentration dependence for increases in intracellular cAMP catalyzed by *B. pertussis* adenylate cyclase were determined by incubation of cardiac cells with varying amounts of the enzyme for 20 min at 37° (Fig. 2). The maximum levels of intracellular cAMP produced were 13,000 pmol of cAMP/mg in the presence of 3.0 units of *B. pertussis* adenylate cyclase/plate. Measurable increases in intracellular cAMP were obtained with as little as 0.01 unit of enzyme/plate which stimulated the synthesis of 1000 pmol of cAMP/plate. It was not technically feasible to use higher concentrations of the enzyme because of the concentration of the adenylate cyclase preparation and limits on the volume of enzyme sample that could be applied. The response to the bacterial enzyme was clearly not saturated at 3.0 units/plate.

Time course of entry of adenylate cyclase in cardiac cells. In the experiments described above, cardiac cells were routinely treated with *B. pertussis* adenylate cyclase for 20 min to maximize synthesis of intracellular cAMP. It was of some interest to monitor the kinetics for cell invasion since bacterial toxins vary considerably in the time required to cross animal cell membranes. Chick cardiac cells were incubated at 37° for various periods of time with *B. pertussis* adenylate cyclase (0.15 unit/plate). At 2, 5, 10, 15, and 20 min the incubation was terminated by removal of the media and then washing with ice-cold PBS (Fig. 3). An increase in intracellular cAMP was observed within 2 min after treatment of cardiac cells with *B. pertussis* adenylate cyclase; however, the rate of cAMP synthesis was not linear over 20 min. The rapid increase in intracellular cAMP caused by *B. pertussis* adenylate cyclase is comparable to that seen with erythrocytes or neuroblastoma cells (17)

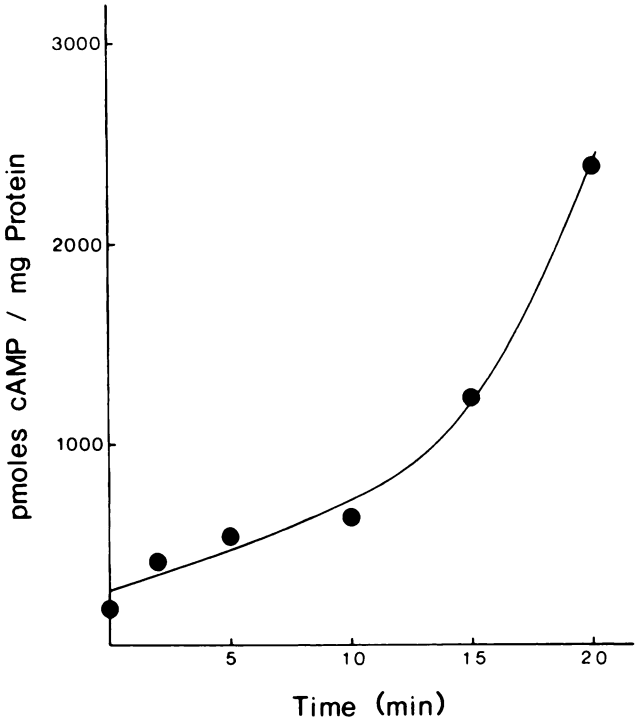

Fig. 3. Time course for increase in intracellular cAMP in cultured chick cardiac cells. Chick cardiac cells were grown to 80–100% confluency as described in Materials and Methods. Cultures were washed with serum-free M199, then preincubated with 5 mM theophylline in serum-free M199 for 20 min at 37°. After preincubation, cells were incubated at 37° with M199 + 5 mM theophylline which contained 0.7 unit of adenylate cyclase activity (units = nmol of cAMP produced/min). The incubations were terminated at the indicated times by removal of the media and washing with ice-cold PBS.

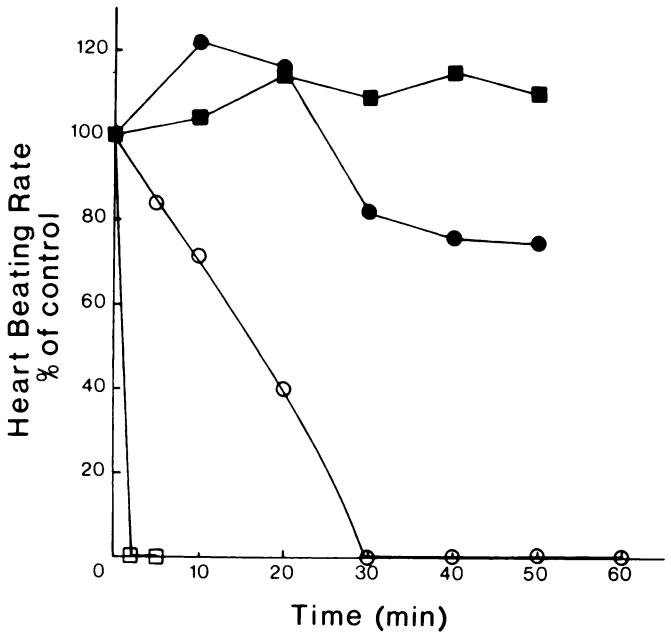

Fig. 4. Effect of adenylate cyclase and forskolin on the beating rate of chick cardiac cell aggregates. Chick cardiac cell aggregates were grown as described in Materials and Methods. Aggregates were washed with Earle's salts, containing 2.7 mM KCl, and allowed to equilibrate for 10 min in a CO_2 incubator. Prior to any additions the time required for 20 beats was measured (100% control). At time zero the following additions were made: ○, 0.2 unit of *B. pertussis* adenylate cyclase (n = 4); ●, 0.2 unit of *B. pertussis* adenylate cyclase + 5.4 μM CaM (n = 2); □, 5.4 μM CaM (n = 2); or ■, 100 μM forskolin (n = 2). All additions were in Earle's salts, containing 2.7 mM KCl, prewarmed to 37°. Incubations were in a CO_2 incubator with the plates removed at indicated times to determine time required for 20 beats; reported as percentage of control.

and suggests that the enzyme may not enter cardiac cells by means of receptor-mediated endocytosis.

Effect of adenylate cyclase on the beating rate of chick cardiac cell aggregates. The beating rates of chick cardiac cell aggregates treated with *B. pertussis* adenylate cyclase were monitored in order to determine whether the increases in intracellular cAMP described above had any effect on the rate of cardiac cell contractions. Chick cardiac cell aggregates were incubated with *B. pertussis* adenylate cyclase (2 units of enzyme/plate), the enzyme plus CaM, CaM alone, or 100 μM forskolin at 37°, and the beating rates were monitored as a function of time for 1 hr (Fig. 4). Beating rates are reported as a percentage of the rate observed with control cells (100 beats/min) which were treated identically without forskolin or *B. pertussis* adenylate cyclase. Cardiac cell aggregates incubated in the presence of *B. pertussis* adenylate cyclase showed a rapid decline in beating rate and stopped beating altogether after 30 min of exposure to the enzyme. Addition of CaM to *B. pertussis* adenylate cyclase almost completely inhibited the effect of the enzyme on cardiac cell beating rate. This effect of CaM is consistent with the data in Fig. 1 which illustrated that the increases in intracellular cAMP caused by *B. pertussis* adenylate cyclase were inhibited by binding of CaM to the enzyme. CaM by itself had no effect on the beating rate of cardiac cell aggregates. Forskolin, at 100 μM, completely inhibited the beating of cardiac cell aggregates, but the response to forskolin was much more rapid and heart cells stopped beating after 2 min of treatment with the drug.

Since the increases in intracellular cAMP catalyzed by 2.0 units of adenylate cyclase/plate were quite high (6000–7000 pmol of cAMP/mg) and completely inhibited beating of cardiac cells, several lower concentrations of enzyme were examined (Fig. 5). *B. pertussis* adenylate cyclase at 1.8 or 0.18 units/plate both inhibited the beating rate of cardiac cells, but the rate of inhibition was faster at the higher enzyme concentration. In contrast, enzyme at 0.018 unit/plate stimulated the beating rate of cardiac cells by 40% and there was no inhibition of beating rate for at least 30 min. This amount of bacterial adenylate cyclase catalyzed an increase in intracellular cAMP of approximately 800 pmol of cAMP/mg relative to untreated controls.

Discussion

Previous studies have established that *B. pertussis* adenylate cyclase invades several types of animal cells including lymphocytes, neutrophils, S49 lymphoma cells, erythrocytes, and neuroblastoma cells (15–17). In this study, treatment of cultured chick cardiac cells with IAP-free *B. pertussis* adenylate cyclase resulted in significant increases in intracellular cAMP that were observable within 2 min after treatment of cells with the enzyme. The maximum increase in intracellular cAMP reported in this study (13,000 pmol of cAMP/mg of cellular protein) was significantly higher than that produced by stimulation of cardiac cells with forskolin, isoproterenol, or forskolin plus isoproterenol. Thus, the increases in intracellular cAMP caused by the bacterial enzyme preparation exceeded the maximum pro-

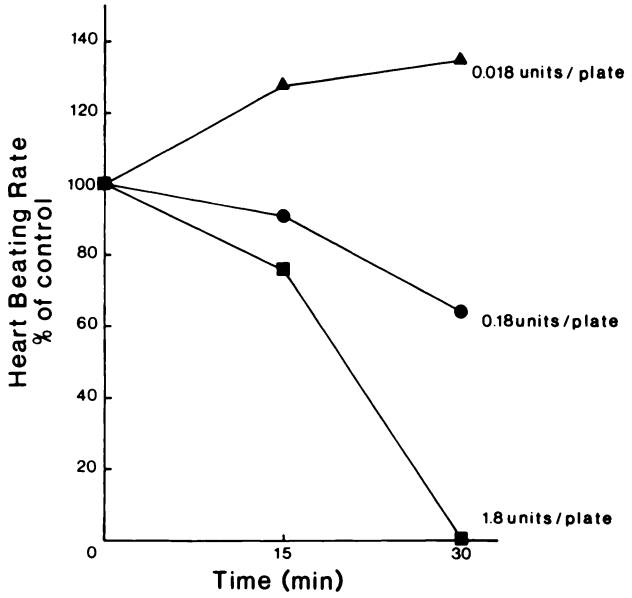

Fig. 5. Effect of varying amounts of *B. pertussis* adenylate cyclase on beating rate of chick cardiac cell aggregates. Chick cardiac cell aggregates were grown as described in Materials and Methods. Aggregates were washed with Earle's salts, containing 2.7 mM KCl, and allowed to equilibrate for 10 min in a CO_2 incubator. Prior to any additions the time required for 20 beats was measured (100% control). At time zero, the indicated amounts of adenylate cyclase were added. All additions were in Earle's salts, containing 2.7 mM KCl, prewarmed to 37°. Incubations were in a CO_2 incubator with the plates removed at indicated times to determine time required for 20 beats; reported as percentage of control.

duction of cAMP catalyzed by the endogenous adenylate cyclase. Furthermore, large increases in intracellular cAMP were obtained in the absence of phosphodiesterase inhibitors, indicating that the internalized bacterial enzyme can catalyze the synthesis of cAMP at rates exceeding that of total endogeneous phosphodiesterase activity.

The increases in intracellular cAMP caused by *B. pertussis* adenylate cyclase were inhibited by the addition of CaM, even though the activity of the enzyme was stimulated by CaM. Apparently, the enzyme · CaM complex cannot enter heart cells. We conclude that *B. pertussis* adenylate cyclase enters heart cells, forms a complex with CaM which activates the enzyme, and catalyzes the formation of cAMP from intracellular ATP. Since CaM stimulation of the enzyme can occur in the absence of free Ca^{2+} (31), activation of the enzyme would not be dependent upon the concentration of intracellular Ca^{2+}. The mechanism for cell entry is undefined; however, the very strong affinity of the enzyme for CaM (31) creates a substantial free energy gradient driving transport of the enzyme into cells since there is no extracellular CaM.

Because contraction of heart muscle is regulated by fluctuations in cAMP (32), one would anticipate that invasion of cardiac cells by the bacterial adenylate cyclase would affect the beating rates of cardiac cell aggregates. Low concentrations of the enzyme, that increased intracellular cAMP approximately 800 pmol of cAMP/mg, did indeed stimulate the beating rate of cardiac cell cultures 30–40%. However, higher concentrations of the enzyme markedly inhibited beating rates. In fact, elevations of intracellular cAMP by 5000 to 6000 pmol of cAMP/mg by *B. pertussis* adenylate cyclase completely inhibited cardiac cell beating after 30 min of exposure to the enzyme. The effects of *B. pertussis* adenylate cyclase on beating rates, like the increases in cAMP, were antagonized by binding of CaM to the enzyme. Forskolin, at 100 μM, also completely inhibited the beating of cardiac cells, but the response to forskolin was much more rapid. Although the mechanism for inhibition of beating caused by *B. pertussis* adenylate cyclase or forskolin is not known, it may be due to extremely high levels of cAMP which are generally toxic for animal cells. Alternatively, high cAMP may stimulate the release of excess intracellular free Ca^{2+}, resulting in contracture. The latter mechanism would be analogous to caffeine contracture which is due to unphysiologically excess caffeine, elevated intracellular cAMP, and excessive Ca^{2+} release (33). There is also the possibility that interactions between the bacterial adenylate cyclase and the cytoplasmic membrane may alter membrane function and affect the heart contractility.

In summary, *B. pertussis* adenylate cyclase invades chick cardiac cells, elevates intracellular cAMP, and alters the beating rate of chick heart cell aggregates. The influence of the enzyme on heart beating rates may be due to changes in intracellular cAMP, although other mechanisms including perturbation of membrane structure and ion permeability have to be considered. The properties of *B. pertussis* adenylate cyclase are consistent with the proposal that this enzyme is a toxin which invades a variety of animal cells and alters cellular functions through increases in cAMP.

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