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Calmodulin Inhibits Entry of *Bordetella pertussis* Adenylate Cyclase into Animal Cells†

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ABSTRACT: *Bordetella pertussis*, the pathogen responsible for whooping cough, releases a soluble calmodulin-sensitive adenylate cyclase into its culture medium. Recently, Confer and Eaton [Confer, D., & Eaton, J. (1982) *Science (Washington, D.C.) 217*, 948–950], as well as Hanski and Farfel [Hanski, E., & Farfel, Z. (1985) *J. Biol. Chem. 290*, 5526–5536], have shown that crude extracts from *B. pertussis* containing adenylate cyclase activity cause elevations in intracellular cAMP when incubated with human neutrophils or lymphocytes. These investigators proposed that the bacterial enzyme enters animal cells and catalyzes the formation of cAMP from intracellular ATP. In this study, *B. pertussis* adenylate cyclase was purified to remove contaminating islet activating protein and examined for its effects on intracellular cAMP levels of human erythrocytes and N1E-115 mouse neuroblastoma cells. In both cases, the enzyme catalyzed the formation of intracellular cAMP. Addition of calmodulin to the adenylate cyclase preparations completely inhibited formation of intracellular cAMP catalyzed by the bacterial enzyme, indicating that cAMP was not synthesized extracellularly and then taken up by the cells. These experiments illustrate that the bacterial enzyme does enter animal cells and that the enzyme–calmodulin complex does not.

The culture medium of growing *Bordetella pertussis* contains a number of biologically active components that may play a role in the pathogenesis of whooping cough (Olson, 1975; Jawetz et al., 1978). One of these, islet activating protein (IAP),[1] has been purified from the culture medium of the bacteria (Yajima et al., 1978a,b; Sekura et al., 1983). 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 (Katada & Ui, 1981, 1982a,b). The attenuation of receptor-mediated inhibition can result in an increase in intracellular cAMP levels.

Another factor, which is thought to play a role in the pathogenesis of whooping cough, is the adenylate cyclase from *B. pertussis*. Adenylate cyclase activity was detected in the culture medium of *B. pertussis* (Hewlett et al., 1976; Hewlett & Wolff, 1976), and the soluble bacterial adenylate cyclase was found to be stimulated by calmodulin (CaM) even though *B. pertussis* does not contain CaM (Wolff et al., 1980). Weiss et al. (1983, 1984) have determined that *B. pertussis* mutants deficient in adenylate cyclase are avirulent. Furthermore, Confer & Eaton (1982) and Hanski & Farfel (1985) have shown that incubation of crude preparations of the *B. pertussis* adenylate cyclase with animal cells causes increases in intracellular cAMP. On the basis of these data, these investigators postulated that the adenylate cyclase from *B. pertussis* enters animal cells and catalyzes the formation of cAMP from intracellular ATP. It is also possible that IAP or another unidentified factor present in the impure adenylate cyclase preparations modulated animal cell adenylate cyclase or cyclic nucleotide phosphodiesterase activities, thereby leading to increased intracellular cAMP.

In this study, we demonstrate that a highly purified *B.*

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[1] Abbreviations: CaM, calmodulin; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; IAP, islet activating protein; cAMP, adenosine cyclic 3′,5′-phosphate; Tris, tris(hydroxymethyl)aminomethane; NAD, nicotinamide adenine dinucleotide; PAGE, polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; ATP, adenosine 5′-triphosphate.

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pertussis adenylate cyclase lacking IAP causes increases in intracellular cAMP with human erythrocytes and neuroblastoma cells. Furthermore, binding of CaM to the enzyme inhibited cell invasion, providing an excellent control for leakage of ATP out of the cell and the formation of extracellular cAMP.

Materials and Methods

Materials

QAE-Sephadex, cyanogen bromide activated Sepharose 4B, and Ficoll–Paque were purchased from Pharmacia. Ultrogel AcA 44 was obtained from Bio-Rad. Fetuin, ATP, cAMP, 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 Bio-Rad. IAP was supplied by List Biologics, and Dulbecco's modified essential medium (DMEM) with high glucose content (4.5 g/L) and fetal calf serum were purchased from Grand Island Biological Co. All other reagents were of the finest available grade from commercial sources.

Methods

Adenylate Cyclase Assay. Adenylate cyclase was assayed at 30 °C by the general method of Salomon et al. (1974) 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-HCl, 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%. Protein concentrations were determined by the method of Peterson (1977).

Intracellular cAMP Determination. Erythrocytes were incubated at 37 °C for various periods of time with phosphate-buffered saline (PBS) or adenylate cyclase preparations in PBS. Cells were immediately centrifuged through Ficoll–Paque and washed twice with PBS. Intracellular cAMP was assayed by the method of Gilman (1970) using purified cAMP-dependent protein kinase. Neuroblastoma cells were treated on tissue culture plates and washed twice with PBS before assaying for cAMP.

Preparation of CaM. CaM was prepared from bovine brain by the procedure of Dedman et al. (1977) as modified by Olwin et al. (1984).

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 Bordetella pertussis. B. pertussis adenylate cyclase was purified by the method of Shattuck et al. (1985). A brief description is presented here. *B. pertussis* (Tohama phase 1) was grown from a 5% inoculum in supplemented Stainer–Scholte medium (Stainer & Scholte, 1971) at 35.5 °C with shaking until OD_{650} = 0.5. Bacterial suspensions were centrifuged, and the culture medium containing adenylate cyclase activity was removed. QAE-Sephadex (500 mL) was added to 18 L of *B. pertussis* culture medium and stirred for 1 h. The resin was poured in 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. $CaCl_2$ and CaM were added to peak I adenylate cyclase to a concentration of 0.2 mM and 1 μM, respectively, and the enzyme was reapplied to a second QAE-Sephadex column. The enzyme was eluted as a single peak (CaM-shifted peak I pool) with a linear 40 mM to 1.2 M NaCl gradient.

The CaM-shifted peak I pool was applied to a 10-mL fetuin–Sepharose column. The protein that flowed through the fetuin–Sepharose column was concentrated 25-fold by Amicon ultrafiltration using a PM-10 membrane. Four milliliters of concentrated material was applied to an Ultrogel AcA 44 column. Fractions were assayed for adenylate cyclase activity and protein. All steps of the purification were carried out at 4 °C.

Adenylate cyclase preparations purified through the first QAE-Sephadex column were also passed over fetuin–Sepharose to remove contaminating IAP.

SDS Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was carried out as described by Laemmli (1970) and stained with Coomassie Brilliant Blue.

Detection of Contaminating Islet Activating Protein. The presence of IAP was detected by its ability to catalyze the ADP-ribosylation of GTP binding proteins in cerebral cortex membranes (Neer et al., 1984; Sternweis & Robishaw, 1984). 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 °C. Bovine brain membranes prepared by the method of Andreasen et al. (1983) were then incubated for 1 h at 30 °C 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-HCl (pH 8), and 20 μCi of [^{32}P]NAD. The reaction was stopped by the addition of 1 mL of cold 100 mM Tris-HCl (pH 8). Membranes were washed and applied to a 10% polyacrylamide slab gel in SDS and autoradiographed to detect labeling of IAP substrates.

Isolation and Purification of Human Erythrocytes. Whole blood, collected in 0.15% EGTA, was centrifuged at 2000 rpm for 5 min, and the upper layer was removed. The erythrocytes were washed twice in PBS containing 5 mM theophylline and then centrifuged through Ficoll–Paque to remove lymphocytes. Packed cells were again washed 2 times in PBS and then resuspended in an equal volume of PBS. Contamination by white blood cells was quantitated by examination of Wright stained cells by phase contrast microscopy. Purified erythrocytes contained less than 50 lymphocytes/10^6 erythrocytes.

Neuroblastoma Cells. N1E-115 mouse neuroblastoma cells (passages 16–26) were grown at 37 °C in DMEM (high glucose) supplemented with 5% fetal calf serum, without antibiotics, in an atmosphere of 10% CO_2/90% humidified air. The cells were grown to 80–100% confluency in plastic tissue culture dishes prior to the start of each experiment. Cells were subcultured weekly, and the culture medium was changed on days 3, 5, and daily thereafter. All experiments were begun on day 6 following subculture.

Results

Purification of CaM-Activated Adenylate Cyclase from B. pertussis. The procedure used for purification of adenylate cyclase is summarized in Table I (Shattuck et al., 1985). Since the adenylate cyclase is released by the bacteria into the culture media, removal of the cells by centrifugation resulted in approximately a 6-fold increase in the specific activity of adenylate cyclase. Application of the soluble CaM-sensitive adenylate cyclase to QAE-Sephadex and elution with a linear NaCl gradient resulted in separation of two forms of adenylate cyclase activity, peak I and peak II. Peak I adenylate cyclase

Table I: Purification of Calmodulin-Activated Adenylate Cyclase from *B. pertussis*

purifícn step[a]	total act. × 10^4 (nmol/min)	% yield of act.	total protein (mg)	sp act. (μmol min^{-1} mg^{-1})	x-fold purifícn
bacteria + culture media[b]	318	100	8.4×10^3	0.38	
culture media	202	63.5	8.3×10^2	2.4	6.3
QAE-Sephadex peak I	19.1	5.9	8.5	22.8	60.0
calmodulin shift[c]	7.9	2.5			
fetuin–Sepharose[c]	6.4	2.0			
Ultrogel AcA 44	2.4	0.75	6.6×10^{-2}	360.6	948.9
Ultrogel AcA 44 peak fraction	0.608	0.2	9.9×10^{-3}	608.1	1600.3

[a] Adenylate cyclase was assayed in the presence of 5 mM $MgCl_2$ and 2.4 μM calmodulin. [b] Cultures were assayed prior to centrifugation. [c] Protein was not assayed due to contaminating free CaM.

had a specific activity of 22.8 μmol of cAMP min^{-1} mg^{-1} and was stimulated 10–30-fold by CaM, with the degree of CaM stimulation varying from one preparation to another.

Further purification of peak I adenylate cyclase activity was obtained by the addition of CaM to the pooled activity and reapplication to QAE-Sephadex. The addition of CaM to the adenylate cyclase activity significantly shifted the elution position of the enzyme. This CaM-induced shift in elution does result in further purification of the enzyme; free CaM, however, eluted at approximately the same conductivity. As a result, the specific activity of the CaM-shifted adenylate cyclase could not be determined due to contaminating levels of free CaM.

Contaminating CaM was removed by gel filtration using Ultrogel AcA 44. Use of this resin resulted in separation of adenylate cyclase activity from the peak of free CaM, which was one of the major sources of contaminating protein at this stage of the preparation. The adenylate cyclase obtained from this column was no longer CaM-sensitive, presumably because CaM was strongly associated with the enzyme. This purification protocol resulted in a 1600-fold purification of adenylate cyclase activity in the peak fraction or 1000-fold purification of pooled activity (Table I). The specific activity of the most highly purified enzyme was 608 μmol of cAMP min^{-1} mg^{-1}, which is higher than any previously reported preparation of adenylate cyclase. When the M_r of 44 300 (Shattuck et al., 1985; Wolff et al., 1983) and a specific activity of 608 μmol of cAMP min^{-1} mg^{-1} are used and one catalytic site per monomer is assumed, the minimum turnover number of the enzyme would be 27 000 min^{-1}. The K_m of the enzyme for ATP was 2.0 mM in the presence of CaM (Shattuck et al., 1985).

Since IAP may increase intracellular cAMP levels under certain conditions, it was necessary to remove IAP from the adenylate cyclase preparations. To ensure complete removal of IAP from the various adenylate cyclase preparations, peak I and CaM-shifted peak I pool were applied to a fetuin–Sepharose column. Fetuin is a serum sialoglycoprotein that binds IAP and has been used in its purification (Sekura et al., 1983); however, adenylate cyclase activity does not absorb to fetuin–Sepharose. In order to determine if IAP had actually been removed from the preparations, bovine brain membranes were labeled with various sources of IAP and [^{32}P]NAD (Figure 1). When pure IAP was incubated with [^{32}P]NAD and bovine brain membranes, two labeled polypeptides with M_r of 39 000 and 41 000 were readily detected on SDS gels. The partially purified adenylate cyclase preparations that had been submitted to fetuin–Sepharose chromatography showed no detactable IAP activity. It is interesting, however, that the enzyme preparation did catalyze the labeling of a high molecular weight polypeptide (M_r approximately 190 000) and that CaM apparently increased labeling of this polypeptide. The identity of this protein is unknown.

Entry of B. pertussis Adenylate Cyclase into Human Erythrocytes. Human erythrocytes were examined for cell entry by the *B. pertussis* adenylate cyclase since they are relatively stable, they have no detectable adenylate cyclase activity, and they have adequate ATP levels to support formation of cAMP. Human erythrocytes were incubated at 37 °C for various periods of time with either peak I adenylate cyclase that had been passed over a fetuin–Sepharose column (129 nmol of cAMP/min per assay) or adenylate cyclase purified through the Ultrogel AcA 44 column (206 nmol of cAMP/min per assay). The peak I adenylate cyclase, which contained no CaM, was stimulated 3.1-fold by exogenous CaM, and the Ultrogel AcA 44 adenylate cyclase was no longer CaM-sensitive, since CaM had been added during the purification. At 5, 15, 25, and 45 min, aliquots were removed and immediately centrifuged through Ficoll–Paque in order to separate any adenylate cyclase not associated with the cells. The cells were washed in PBS and assayed for cAMP. Peak I adenylate cyclase purified through the fetuin–Sepharose column caused a substantial increase in cAMP levels to 375 pmol of cAMP/10^9 cells, which was at least a 30-fold increase over cells incubated with adenylate cyclase purified through Ultrogel AcA 44 (Figure 2). Furthermore, the increase in cAMP was very rapid, with maximum levels reached after 15 min. There was no detectable lag phase for cAMP formation, and increases in cAMP were detectable within 2–3 min after incubation of enzyme with erythrocytes.

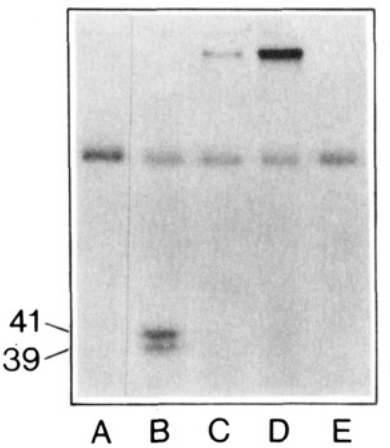

FIGURE 1: Assay for islet activating protein contamination of adenylate cyclase preparations. Bovine brain membranes were incubated with [^{32}P]NAD (20 μCi per sample), 10 mM thymidine, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM ATP, 10 μM NAD, and 40 mM Tris-HCl (pH 8.0) in the presence of (A) no additions, (B) 8 μg of pure islet activating protein, (C) 10 μg of *B. pertussis* adenylate cyclase purified through QAE-Sephadex and fetuin–Sepharose chromatography (adenylate cyclase peak I), (D) condition C with 50 μg of added CaM present, and (E) 5 μg of adenylate cyclase purified through Ultrogel AcA 44 chromatography. Samples were then run on SDS–PAGE (12.5%) and audioradiographed to detect ADP-ribosylated polypeptides.

There are two possible reasons why the highly purified

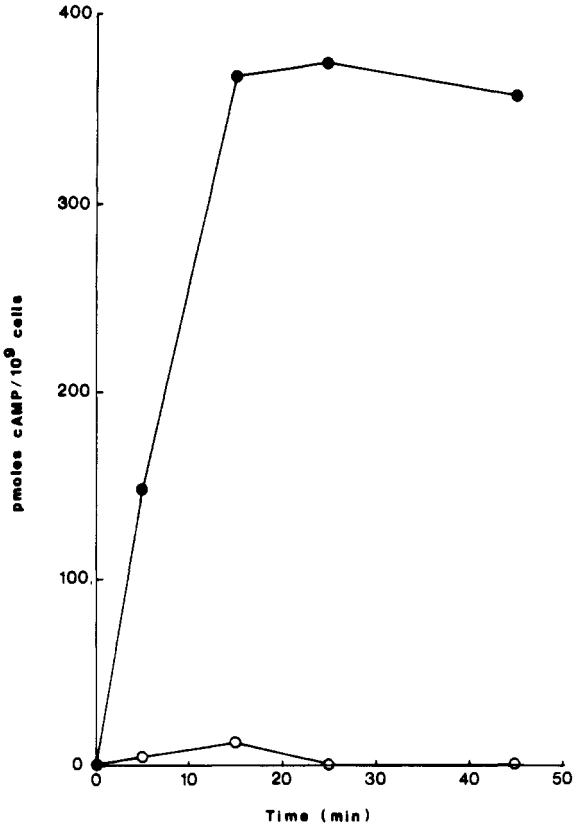

FIGURE 2: Time course for increase in intracellular cAMP in human erythrocytes. Human erythrocytes (prepared as described under Methods) were incubated at 37 °C with peak I adenylate cyclase that had been passed over fetuin–Sepharose (●) or Ultrogel AcA 44 (○) adenylate cyclase at 37 °C. Samples containing 2.6×10^9 cells were removed at the indicated times, centrifuged through Ficoll–Paque, and washed with PBS. Packed cells were assayed for intracellular cAMP. The adenylate cyclase activity for peak I was 129.3 nmol of cAMP min^{-1} mL^{-1} and for Ultrogel AcA 44 adenylate cyclase was 206.3 nmol of cAMP min^{-1} mL^{-1}.

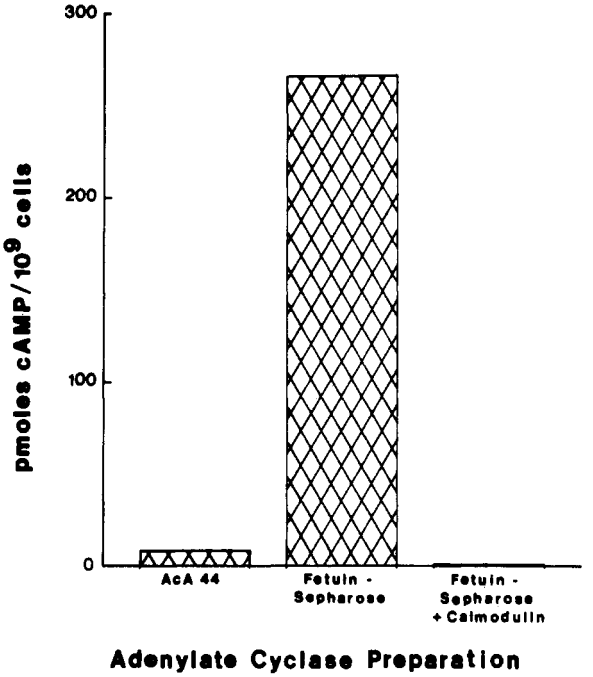

FIGURE 3: Inhibition of intracellular cAMP increase by CaM. Human erythrocytes (2.6×10^9 cells/mL) were incubated with peak I adenylate cyclase that had been passed over fetuin–Sepharose, fetuin–Sepharose adenylate cyclase with 5.4 μM CaM, or Ultrogel AcA 44 adenylate cyclase for 10 min at 37 °C. Erythrocytes were centrifuged through Ficoll–Paque, and the packed cells were assayed for intracellular cAMP.

adenylate cyclase from the Ultrogel AcA 44 column was unable to increase intracellular cAMP levels in erythrocytes. Since this preparation was more highly purified than peak I adenylate cyclase purified through fetuin–Sepharose, it is possible that a component which facilitates cell entry of the adenylate cyclase was separated from catalytic activity. Alternatively, the more highly purified enzyme had CaM complexed to it, which may have inhibited penetration of the enzyme into erythrocytes. Therefore, CaM was added to the enzyme purified through fetuin–Sepharose, and the CaM–enzyme complex was assayed for its effects on cAMP levels of erythrocytes (Figure 3). The adenylate cyclase activities used in the incubations with fetuin–Sepharose adenylate cyclase, fetuin–Sepharose + CaM, and the Ultrogel AcA 44 purified adenylate cyclase were 82.6, 99.4, and 27.1 nmol/min per assay, respectively. Addition of CaM to the enzyme completely inhibited the formation of intracellular cAMP catalyzed by the enzyme, indicating that the enzyme–CaM complex does not enter erythrocytes. Although this experiment shows inhibition of enzyme entry into cells by CaM, the existence of a protein subunit that facilitates cell entry of the enzyme cannot be ruled out. The observation that CaM completely inhibited intracellular cAMP increases caused by the bacterial enzyme indicated that cAMP was not synthesized extracellularly and then taken up by the cells since there was equivalent of higher adenylate cyclase activity present extracellularly when CaM was complexed to the enzyme.

Entry of B. pertussis Adenylate Cyclase into Neuroblastoma Cells. It was of some interest to determine if the adenylate cyclase could enter other types of animal cells. Therefore, neuroblastoma cells, which had been washed in DMEM with 5 mM theophylline, were incubated with either DMEM, adenylate cyclase purified through fetuin–Sepharose, or the enzyme with added CaM. All incubations were in the presence of 5 mM theophylline. Again, an increase in intracellular cAMP levels was seen only when the enzyme was present without CaM (Figure 4). After a 10-min incubation with the enzyme, the intracellular cAMP was 715.7 pmol of cAMP per plate, whereas the cells treated with DMEM or enzyme with CaM contained 110 pmol of cAMP per plate. Intracellular cAMP levels were also increased in the absence of theophylline; however, the maximum levels were augmented by the addition of theophylline (Table II). Although both erythrocytes and neuroblastoma cells were susceptible to invasion by the *B. pertussis* enzyme, cell entry was apparently more effective with the neuroblastoma cells since the maximum production of cAMP was approximately 0.4 pmol of cAMP/10^6 erythrocytes or 300–800 pmol/10^6 neuroblastoma cells. Alternatively, erythrocytes may contain higher levels of an enzyme that inactivates or attenuates the activity of *B. pertussis* adenylate cyclase once it enters the cell.

DISCUSSION

We have presented evidence that the adenylate cyclase from *B. pertussis* is capable of entering erythrocytes and neuroblastoma N1E-115 cells where it catalyzes the formation of cAMP from intracellular ATP. Although the efficiency of the process was quite low with erythrocytes compared to neuroblastoma cells, erythrocytes served as an excellent model system because they contained no measurable adenylate cyclase activity. Therefore, we assume that any intracellular cAMP formed was due to the bacterial enzyme encountering intracellular ATP. Since the binding of CaM to the enzyme

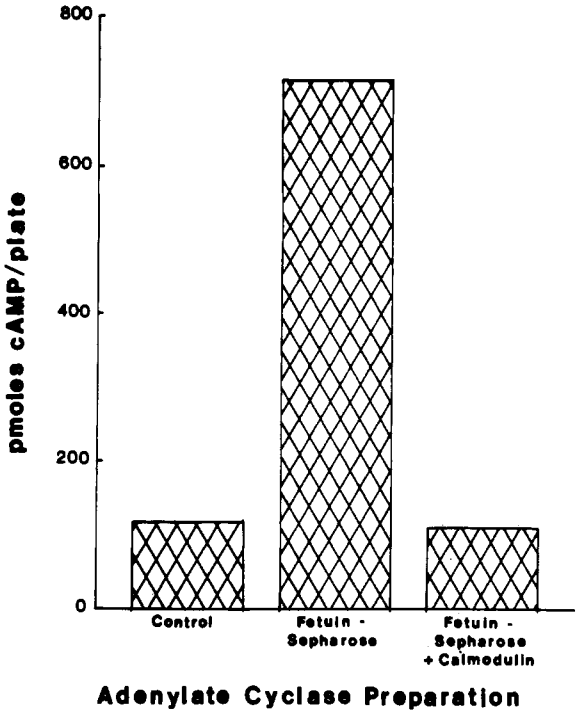

FIGURE 4: Increase in intracellular cAMP levels in N1E-115 cells. Mouse neuroblastoma cells were grown to confluency as described under Methods. Cultures were washed with serum-free DMEM and then preincubated with 5 mM theophylline in serum-free DMEM for 30 min at 37 °C. After preincubation, cells were incubated with DMEM plus 5 mM theophylline that contained peak I adenylate cyclase (fetuin–Sepharose), peak I adenylate cyclase with 5.4 μM CaM (fetuin–Sepharose + calmodulin), or no addition (control) for 10 min at 37 °C.

Table II: Increase in Intracellular cAMP Levels in N1E-115 Cells in the Presence and Absence of Theophylline

treatment[a]	pmol of cAMP per plate
buffer	98 ± 40
buffer + 5 mM theophylline	71 ± 7
adenylate cyclase	1041 ± 449
adenylate cyclase + 5 mM theophylline	2378 ● 270

[a] Cultures were washed with serum-free DMEM and then preincubated with either buffer (Dulbecco's modified essential medium) or buffer with 5 mM theophylline for 30 min at 37 °C. After preincubation cells were treated as indicated. Incubations were carried out at 37 °C for 10 min. Cells were then washed with PBS and assayed for cAMP. The adenylate cyclase preparation used was the enzyme purified through fetuin–Sepharose.

inhibited intracellular cAMP increases, it appears that the enzyme–CaM complex cannot cross the membrane. Thus the equilibrium for transfer across the membrane, assuming that it is a reversible process, would energetically favor cell entry because of the high concentrations of CaM found inside the cell.

Hanski & Farfel (1985) reported that their *B. pertussis* adenylate cyclase preparation did not invade erythrocytes. These investigators reported a M_r of approximately 190 000 for the activity that caused cAMP increases in lymphocytes, and they demonstrated that the majority of the adenylate cyclase activity could be separated from the activity causing an increase in intracellular cAMP by gel filtration. The adenylate cyclase used in our study had a M_r of 44 300 (Shattuck et al., 1985). It is also notable that the kinetics for cell entry were quite rapid compared to those reported by Hanski & Farfel (1985). Furthermore, the adenylate cyclase preparation used in our study was considerably more active than that used by Hanski and Farfel; this may explain why we were able to observe entry into erythrocytes and they were not.

The neuroblastoma cells contain adenylate cyclase activity, and it has been demonstrated that IAP can increase intracellular cAMP levels of whole animal cells (Hsia et al., 1984). Therefore, it was necessary to remove contaminating IAP from the *B. pertussis* adenylate cyclase before it could be unambiguously established that the adenylate cyclase was responsible for intracellular cAMP increases. IAP-free *B. pertussis* adenylate cyclase did indeed increase intracellular cAMP levels in both the presence and absence of theophylline, and like erythrocytes, CaM inhibited adenylate cyclase entry into neuroblastoma cells.

B. pertussis produces two different toxins that both increase intracellular cAMP levels. Although this may seem redundant, there are important differences between the two toxins, and quite clearly a combination of the two may be more effective in raising intracellular cAMP than either toxin alone. One obvious difference between IAP and adenylate cyclase is the kinetics of cell invasion. Adenylate cyclase elevated intracellular cAMP levels within 2 min after exposure to cells, whereas IAP is much slower acting and its effects are not evident until 40–60 min after addition. Thus, adenylate cyclase may be responsible for rapid, short-term increases in cAMP while the slower acting IAP may amplify cAMP increases caused by adenylate cyclase. It is also possible that the two toxins have different cell surface receptors and produce their major effects on different types of cells.

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Registry No. cAMP, 60-92-4; adenylate cyclase, 9012-42-4.

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Articles

Differences in the Condensation of Chromatin by Individual Subfractions of Histone H1: Implications for the Role of H1° in the Structural Organization of Chromatin†

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ABSTRACT: The effectiveness of histone H1 subfractions H1-1 and H1° in inducing the ordered condensation of chromatin was examined by thermal denaturation, circular dichroism, electric birefringence, orientation mechanism, and orientational relaxation time measurements. Soluble rat liver chromatin was stripped of H1 by dissociation in 500 mM NaCl and long fragments of chromatin were subsequently reassociated with purified individual H1 subfractions for ratios of 1 and 2 mol of H1 per nucleosome. H1 subfractions behave differently with respect to their interactions with DNA in chromatin: although the orientation mechanisms of reconstituted chromatins are identical, H1° induces a less efficient protection of DNA than H1-1, as shown by nuclease digestion and by the length of free extended linker DNA determined by electric birefringence. This corresponds to a more extended structure of H1°-reconstituted chromatin as judged by the value of relaxation time. One can imagine that the replacement of H1 by H1° leads to a different structure or stability of the chromatin, confering a certain degree of flexibility of this region. This may be related to the functional role of H1° in DNA replication or transcription and may explain metabolic and evolutionary differences among H1 subfractions as recently suggested by Lennox [Lennox, R. W. (1984) *J. Biol. Chem. 259*, 669-672]. The extent of condensation when H1-depleted chromatin is overloaded with histones is probably a function of the electrostatic interactions between the basic C-terminal tails of histones and chromatin. Electric birefringence also reveals differences between native and reconstituted chromatins that are overlooked by several other criteria.

It is now widely accepted that histone H1 is involved in the maintenance and modulation of higher order structure in chromatin (Littau et al., 1965; Bradbury et al., 1973; Billett & Barry, 1974; Noll & Kornberg, 1977; Thoma et al., 1979). In fact, the class of lysine-rich histone H1 found in most eucaryotic cells is the most variable of the five histone classes. It shows both tissue and species specificity (Bustin & Cole, 1968; Kinkade, 1969; Panyim & Chalkley, 1969), and this heterogeneity in the primary structure of H1 subfractions may play a role in the degree of chromatin condensation (Huang & Cole, 1984). Despite their common structural organization, previous studies have suggested that H1 histones differ among themselves with respect to their interactions with DNA (Gorka & Lawrence, 1979; Welch & Cole, 1979, 1980; Liao & Cole, 1981a,b). It is therefore conceivable that the structural diversity of H1 subfractions is related to their potential for functional diversity. Among H1 histones, the fraction H1° could be involved in cell proliferation (Pehrson & Cole, 1980) or rather in terminal differentiation (Gjerset et al., 1982). When H1° is present, it replaces H1 on the linker region of

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