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Extracellular cAMP formation from host cell ATP by *Bordetella pertussis* adenylate cyclase

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The effect of exogenously added adenylate cyclase from *Bordetella pertussis* (strain 114) has been investigated in Y-1 mouse adrenal tumor, chinese hamster ovary (CHO) and several other cells. A partially purified adenylate cyclase was found not to enter cells but, nevertheless, produced large amounts of cAMP in the medium. We could show that this resulted from release of ATP (and not larger molecules). The ATP released by the cells could be (1) directly measured and was replenished after each change of medium; (2) was reciprocally related to the cAMP produced; and (3) was competed for by ATPases present in added serum or by hexokinase and, less effectively, by exoenzymes on the cell surface. The extent of ATP leakage varied widely between different cell lines, being marked in CHO and Y-1 adrenal cells but negligible in transformed lymphocyte lines. The uncertainty of the origin of cAMP found in media of cultured cells requires separate analysis of cell and medium cAMP and an assessment of ATP leakage.

Introduction

Virulent *Bordetella* species secrete adenylate cyclase(s) into the periplasmic space and culture medium. The enzymes differ from most eukaryotic cyclases, are activated by calmodulin [1] and some forms may attain specific activities approaching 1 mmol/min per mg [2–4]. Current evidence suggests that at least one form of the enzyme is a virulence factor and the extracellularly added cyclase enters host cells where it produces high levels of cAMP that lead to a modification of various cellular functions [5–11]. During attempts to study the mode of entry of this cyclase into host cells, it became apparent that large accumulations of cAMP were present in the culture medium of the cells. This suggested to us that studies on the invasiveness of the extracellular *B. pertussis* adenylate cyclase would have to deal with at least two contingencies: (1) The enzyme enters host cells in tissue culture experiments and produces cAMP which then leaks out into the culture medium; (2) ATP leaks out of the tissue culture cells, either spontaneously or induced by the enzyme preparation, and becomes the external substrate for the extracellularly located cyclase. Preliminary experiments had shown that the cruder preparations of the cyclase belonged to category (1), whereas purification of the catalytic activity was accompanied by loss in invasiveness, but re-

Abbreviations: BSA, bovine serum albumin; CHO, chinese hamster ovary; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; IBMX, isobutylmethylxanthine; MEM, minimum essential Eagle's medium; buffer A, 30 mM Tris-HCl buffer (pH 7.9) plus 1 mM $MgCl_2$; buffer B, 40 mM Tris-HCl buffer (pH 7.9) plus 1 mM $MgCl_2$; Y-1, mouse adrenal cortex tumor; ^{125}I-TME-scAMP, ^{125}I-labelled O^2-monosuccinyladenosine-3′:5′-cyclic monophosphate tyrosyl methyl ester.

tention of the ability to generate cAMP in the extracellular compartment. We took advantage of this loss and used the partially purified adenylate cyclase of *B. pertussis* (strain 114) to study the release of ATP from a variety of cells in culture.

Materials and Methods

Methods

Adenylate cyclase preparation. Bordetella pertussis organisms (strain 114) were grown for 24 h in Stainer-Scholte medium as previously described [2,3]. The cells were harvested by centrifugation and the paste was either stored frozen at −30 °C or was utilized directly for urea extraction. The wet paste was extracted with 4 vols. of 4.0 M freshly prepared urea in buffer B with four 30-s bursts of an omnimixer (at 4 °C). The homogenate was allowed to stand for 10 min with a small amount of DNAase and was centrifuged for 20 min at 23000 × *g* (max) at 4 °C. The pooled pellets were reextracted as above and the supernatant solutions were pooled as the urea extract and stored frozen in liquid N_2.

The urea extract, dialyzed against buffer A was applied to a diethylaminoethyl cellulose (DE52) column (15 × 2.5 cm) equilibrated with buffer A and washed with this buffer until no protein eluted. A NaCl gradient of 0–300 mM in buffer was then started (400 ml), collected in 4-ml fractions at a rate of approx. 1.0 ml · min^{-1}. The major activity peak eluted at conductivities of 2.5–5.1 mohm · cm^{-1}. Peak fractions were pooled and yielded specific activities of adenylate cyclase of 0.035–0.39 μmol cAMP · min^{-1} · mg^{-1} for basal activity and 0.62–4.3 μmol cAMP · min^{-1} · mg^{-1} in the presence of 0.1 μM calmodulin and 10 μM added $CaCl_2$. Aliquots were stored in liquid N_2 (subsequently named DE adenylate cyclase).

The amounts of adenylate cyclase activity added to culture dishes are expressed as nmol cAMP/min per dish as assayed under our optimized conditions including 0.1 μM calmodulin (see below). It must be emphasized that the catalytic activity in the culture medium is substantially less because of lower pH, higher than optimal Ca^{2+} concentrations, lower ATP concentrations and only contaminating concentrations of calmodulin [7].

Adenylate cyclase was measured at 30 °C for 10 min in a total volume of 60 μl containing 60 mM Tris-HCl buffer (pH 7.9), 2 mM $MgCl_2$ and 1 mM ATP (sometimes 10 mM $MgCl_2$ and 5 mM ATP), approx. 0.5 μCi [α-^{32}P]ATP, 10 μM added $CaCl_2$ and 0.1 μM bovine brain calmodulin. The subsequent analysis for [^{32}P]cAMP was determined according to modifications of the method of Salomon et al. [12].

Protein was assayed by the method of Bradford for column profiles [13], otherwise by the bicinchoninic acid method [14].

Cells. Chinese hamster ovary (CHO) cells, kindly provided by Dr. April Robbins (NIH), were grown in 6-cm dishes in minimal essential Eagle's medium with Earle's balanced salt solution (MEM) fortified with nonessential amino acids, 10% fetal calf serum, glutamine, penicillin and streptomycin. They were used before confluence was reached.

Y-1 mouse adrenal tumor cells and a kinase-deficient mutant (Kin-8) [15] (generously provided by Dr. Bernard Schimmer, Toronto) were grown in 6-cm dishes in Ham's F-10 medium containing 10% horse serum, 2.5% fetal calf serum and glutamine, penicillin and streptomycin and were used before they became confluent.

U937 cells, a histiocytic lymphoma [16], and THP-1 cells, a monocytic leukemia [17], were kindly provided by Dr. C.F. Perno and cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum, at 37 °C in 95% air/5% CO_2 humidified atmosphere. The cells were used when they attained a density of $5 \cdot 10^{-5}$/ml.

A fresh blood sample was collected in 0.15% EDTA (w/v), centrifuged 10 min at 400 × *g* at 20 °C in a Sorvall centrifuge, washed three times in 2 vol. of warm serum-free Hanks' MEM, 5 mM theophylline and finally layered onto 0.8 vol. of Ficoll-Hypaque (Pharmacia Fine Chemicals) and centrifuged at 400 × *g* for 40 min at 20 °C. Cells were resuspended in 4 vol. of warm serum-free Hanks' MEM, 5 mM theophylline and incubated in 6-cm petri dishes at 37 °C.

cAMP determinations. CHO and Y-1 cells were washed once with medium for 15 min and then incubated at 37 °C with the cyclase in 3.0 ml culture medium in the presence of 0.3 mM IBMX (final ethanol concentration = 1%). Supernatants

were collected in tubes containing 60 mM acetic acid – 2 mM EDTA for F-10 or 4 mM EDTA for MEM – and kept frozen.

Dishes were rapidly washed twice in cold serum-free medium and immediately frozen on dry ice. The cells were subsequently scraped into cold medium containing 60 mM acetic acid and 2 or 4 mM EDTA and sonicated for 15 s on ice. 500-μl aliquots were then precipitated in 66% ethanol at $-20\,^{\circ}$C for 1 h and centrifuged for 15 min at $1800 \times g$ at $4\,^{\circ}$C; the supernatants were dried in a speed-vacuum concentrator and reconstituted in 50 mM acetate buffer (pH 4.7), 1 mM EDTA, 0.1% BSA for cAMP determination by radioimmunoassay, using a modification of the method described by Brooker et al. [18], using about 10000 cpm/min of ^{125}I-TME-scAMP and anti-cAMP goat IgG was added. After incubation for 3 h at room temperature or overnight at $4\,^{\circ}$C, 30 μl of pansorbin cell suspension pre-absorbed for 30 min at room temperature with a rabbit IgG fraction of anti-goat IgG. After 30 min at room temperature, 1 ml of assay buffer without albumin was added, mixed and centrifuged for 30 min at $1800 \times g$ at $4\,^{\circ}$C. Supernatants were then aspirated and pellets were counted in a scintillation gamma-counter. Hypotonic lysis yielded high values of cAMP in the cell lysate that might erroneously be ascribed as deriving from intracellular production, and could not be used.

U-937 and THP-1 cells were centrifuged at $20\,^{\circ}$C ($400 \times g$ for 10 min), washed, incubated as indicated, washed twice in cold RPMI 0.5 mM IBMX, 4 mM EDTA (pH 7.4), and resuspended in RPMI 60 mM acetic acid, 4 mM EDTA, sonicated for 15 s on ice and treated as described above. Red blood cells were separated on 2 ml of Ficoll-Hypaque, washed, incubated as indicated and washed twice by centrifugation at $400 \times g$ for 40 min at $4\,^{\circ}$C and treated as described above. All cAMP data are averages of duplicate determinations on two or three samples.

ATP measurements. The ATP determinations were performed by a luciferin-luciferase assay kit according to the suppliers instructions. ATP concentrations in cell media were determined against an ATP standard containing the same percentage of culture medium and with an internal standard as an additional correction for possible quenching and interfering effects in cell media. For total intracellular ATP determinations, dishes were extracted on ice with 0.5 M $HClO_4$, for 30 min. The extraction solution was centrifuged at $1800 \times g$ for 10 min. Adherent cells were dissolved in 1 M NaOH and this solution was then added to dissolve the pellet of dislodged cells in the $HClO_4$ extract. A 10-fold dilution of this material in water was used for protein determination by the Bio-Rad reagent [13]. The extraction solution was brought to neutrality with concentrated K_2CO_3. After removing $KClO_4$, ATP concentrations were determined against a standard processed in the same way. A Picolite Luminometer was used, kindly supplied by Dr. C. Londos. All ATP data are averages of duplicate determinations for two or three dishes.

Materials

Reagents used were obtained as follows: urea enzyme grade from Bethesda Research Laboratories, DEAE cellulose type DE-52 from Whatman, ATP type II-S, DNAase, 3-isobutyl-1-methylxanthine, hexokinase, creatine kinase from Sigma; forskolin from Calbiochem and prostaglandin E_2 from Upjohn were used from stock solutions in ethanol, bovine serum albumin was fraction V, ACTH 1-24 was from Ciba, [α-^{32}P]ATP, spec. act. 600–800 Ci/mmol was from New England Nuclear; calmodulin was prepared from bovine brain as described [1]; luciferase and luciferin were from United Technologies, Packard or Los Alamos Diagnostics, Los Alamos, NM; Pansorbin from Behring Diagnostics; ^{125}I-TME-scAMP mono (2.0 mCi/μg) spec. act. above 2000 μCi/μg was from Meloy Laboratories or RPI; anti-AMP antibodies (goat) were from RPI; rabbit anti-goat IgG (IgG fraction) was from Miles Laboratories, Elkhart, IN.

Results

Cyclic AMP in media

Substantial production of extracellular cAMP occurred in all experiments containing extracellular DEAE-purified adenylate cyclase in simple media. This is demonstrated in Table I, which also shows the absence of change in cellular cAMP concentration upon treatment with adenylate

TABLE I

cAMP PRODUCTION IN Y-1 AND CHO CELLS WITH VARIOUS ACTIVATORS

Y-1 cells were incubated for 40 min at 37°C in serum-free F-10 medium, CHO cells were incubated for 60 min at 37°C in serum-free MEM. DE, DEAE-purified adenylate cyclase.

Cell type	Activators (concn.)	Cell (pmol cAMP/ mg protein)	Media (pmol cAMP)
Y-1	control	35	11
	ACTH (2 μM)	488	2400
	forskolin (10 μM)	116	169
	DE (21.4 nmol/min)	33	660
CHO	control	21	12
	forskolin (100 μM)	1854	164
	DE (3.8 nmol/min)	28	1044

cyclase. In ten other experiments with Y-1 cells, the ratio of intracellular cAMP in the presence of DEAE-purified adenylate cyclase to control dishes averaged 1.097 ± 0.071. On the other hand, there was always a large cAMP accumulation in the culture medium. By contrast ACTH and forskolin produced increased cellular cAMP levels in Y-1 cells. These agents also produced high extracellular cAMP concentrations. It is well known that Y-1 cells secrete substantial amounts of cAMP [19] and the data of Table I are consistent with this observation. With CHO cells under forskolin stimulation, a much smaller fraction of cAMP escaped to the medium, whereas adenylate cyclase treatment produced no change in cellular cAMP, but a large accumulation of extracellular cAMP.

Extracellular cAMP accumulation is dose-dependent for added DEAE-purified cyclase in both Y-1 and CHO cells as shown in Fig. 1A for a 1-h exposure; the cAMP production leveled off at high cyclase concentrations. This suggested that another factor probably becomes rate limiting. The accumulation of cAMP in the medium of Y-1 cells exposed to low concentrations of adenylate cyclase preparations shows a linear increase with time over a period of 1 h. Thereafter, it plateaus (Fig. 1B). Similar time curves are obtained with CHO cells (see Fig. 2). Above approx. 1 mg cell protein/ml there is a marked dependence of the extracellular cAMP concentration on the density of Y-1 cells in the dish (Fig. 1C).

A number of other cell types also showed little or no translocation of DEAE-purified adenylate cyclase to their interiors as judged by the lack of response of cellular cAMP (Table II). As in the case of Y-1 and CHO cells, Kin-8 cells and erythrocytes showed a brisk increase in extracellular cAMP in response to DE adenylate cyclase. Interestingly, the two lymphocyte lines showed practically no increase in extracellular or intracellular cAMP, although their own adenylate cyclase responded well to prostaglandin stimulation.

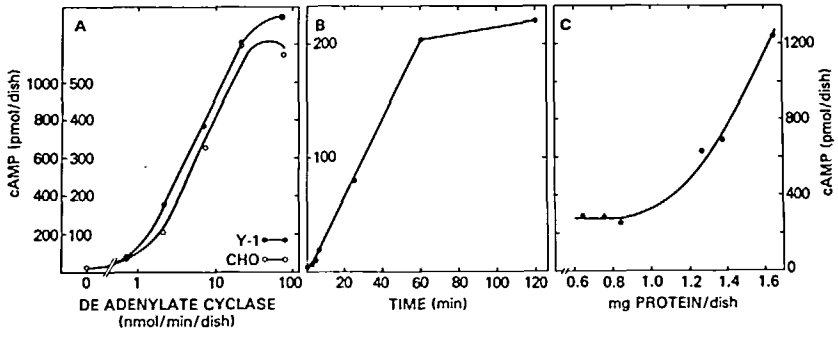

Fig. 1. (A) Dose-curve of cAMP production in the medium in response to DEAE-purified adenylate cyclase. (●) Y-1 cells in serum-free F-10 medium for 1 h at 37°C; proteins, 1.65 ± 0.02 mg/dish (○) CHO cells in serum-free MEM for 1 h at 37°C; proteins, 0.23 ± 0.01 mg/dish. The scale outside the box refers to Y-1 cells, that inside the box refers to CHO cells. (B) Time-curve of cAMP production in the medium of Y-1 cells after DEAE-purified adenylate cyclase addition – serum-free F-10 medium; DEAE-purified adenylate cyclase, 2.1 nmol/min; proteins, 1.78 ± 0.04 mg/dish. (C) cAMP production in the medium of Y-1 cells in response to DEAE-purified adenylate cyclase versus cell density, as measured by the protein content per dish – serum-free F-10 medium for 1 h at 37°C, DEAE-purified adenylate cyclase, 21.4 nmol/min.

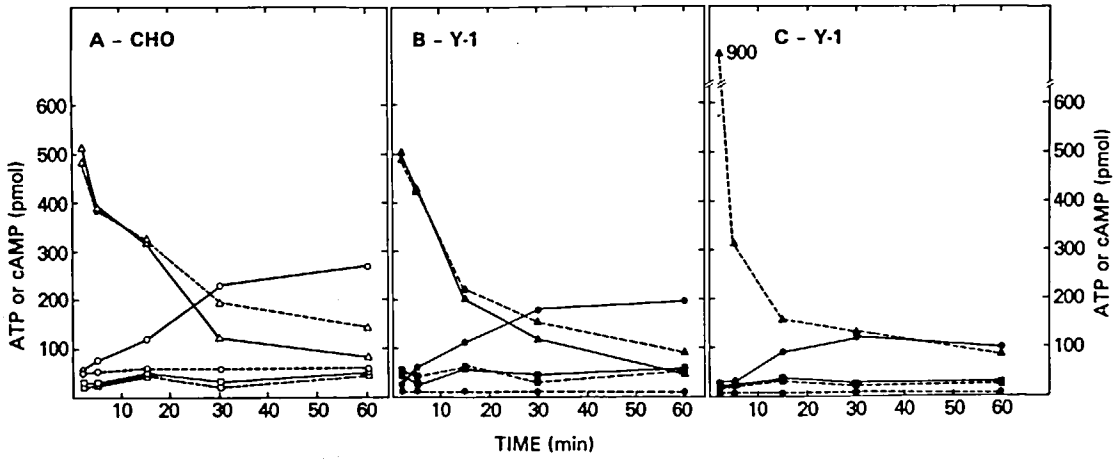

Fig. 2. Time-course of ATP and cAMP concentration in the culture media or in cells after addition of new medium with or without DEAE-purified adenylate cyclase: open symbols, CHO cells; closed symbols, Y-1 cells; triangles represent ATP concentrations (pmol/dish); circles represent cAMP concentrations in culture media (pmol/dish); squares represent intracellular cAMP concentrations (pmol/mg protein); dashed lines indicate control dishes; continuous lines indicate dishes exposed to 14.3 nmol/min of DEAE-purified adenylate cyclase. Incubations were conducted at 37 °C. Time zero is defined as the time of addition of new medium with or without adenylate cyclase. (A) CHO cells in serum-free MEM; proteins, 0.16 ± 0.03 mg/dish; intracellular total ATP 82.8 ± 1.72 nmol/mg protein. (B) Y-1 cells in serum-free F-10 medium proteins, 0.35 ± 0.02 mg/dish; intracellular total ATP, 49.09 ± 0.98 nmol/mg protein. (C) Y-1 cells in serum-free F-10 medium; proteins, 0.46 ± 0.07 mg/dish; intracellular total ATP, 10.01 ± 0.48 nmol/mg protein.

ATP leakage

The following reasons led us to suspect that the source of the extracellular cAMP was extracellular ATP released by the cells: (1) The DEAE form of adenylate cyclase, in contrast to other forms did not penetrate these cells (Tables I and II); and (2)

TABLE II

DE ADENYLATE CYCLASE-INDUCED cAMP FORMATION IN VARIOUS CELL TYPES

Cell type (origin)	cAMP in cells (pmol per mg protein)			cAMP in media (pmol per cell sample)		
	control	DE [a]	PGE_2 (5 μM)	control	DE [a]	PGE_2(5 μM)
Kin-8 [b] (mouse adrenal) (per mg)	32.0	46.0	–	3.0	945	
U-937 [c] (histiocytic lymphoma) (per 10^6 cells)	1.7	1.4	178	0.7	3.2	23
THP-1 [d] (monocytic leukemia) (per 10^6 cells)	9.7	11.0	460	2.4	3.8	49
Human [e] red blood cells (per 10^9 cells)	1.4	1.9	–	3.61	687	–

[a] DEAE-purified adenylate cyclase 21.4 nmol/min.
[b] Cells seeded in 6 cm dishes with 3 ml of serum-free F-10 medium; proteins 1.47 ± 0.01 mg/dish.
[c] $5.5 \cdot 10^5$ cells suspended in 1 ml of serum-free RPMI 1640.
[d] $5.2 \cdot 10^5$ cells suspended in 1 ml of serum-free RPMI 1640.
[e] $1.2 \cdot 10^9$ cells suspended in 1 ml of serum-free Hanks' MEM.

TABLE III

ATP APPEARANCE IN THE MEDIUM OF Y-1 CELLS AND CHO CELLS UPON REPEATED CHANGES OF MEDIUM

Medium ATP was measured at 2 min (initial) or 60 min (final) after addition of fresh medium.

	ATP pmol/dish		
	1st change	2nd change at 15 min	2nd change at 60 min
Y-1			
Initial	900	691	727
Final	86	99	160
CHO			
Initial	479	470	394
Final	140	188	190

a number of cells and tissues are known to leak ATP [20–24]. To this end, we studied the release of ATP from the cells used here and some factors that determine its availability.

Direct measurement of ATP by the luciferase method revealed that 2 min after a medium change, substantial quantities of ATP had accumulated in the medium and repeat changes of medium with the same cells led to repeated ATP release in both Y-1 and CHO cells (Table III). With continued incubation this level dropped markedly, presumably due to the presence of exoATPases on the cell surface [22,25,26].

Time courses of ATP levels after medium changes are listed in Fig. 2 for CHO and Y-1 cells. There was a time-dependent decrease of the ATP concentration in the medium that was reciprocally related to the increase in cAMP generated (Figs. 2A and B). Note that cellular cAMP concentrations remained consistently very low over the 1-h interval. The level of ATP fell only slightly faster in the presence of adenylate cyclase than in control dishes, suggesting that significant degrees of ATP metabolism by exoenzymes on CHO cells must also have occurred, and that ATP continued to be released. The amounts of cAMP measured 1 h after exposure to DE appeared to be a function of the total ATP content of the cells. Thus, when the ATP content of Y-1 cells was reduced from 45 to 10 nmol/mg by partial starvation (fewer medium changes during growth), only about one half the amount of cAMP was found (Fig. 2C). Similar reductions in cyclase-stimulated cAMP yields could be obtained with 0.5 mM dinitrophenol or 5 μg/3 ml antimycin A. These agents had no effect on the adenylate cyclase activity per se at these concentrations. We interpret these cAMP decreases to mean decreased ATP leakage. By the same token, the absence of extracellular cAMP production in the U-937 and THP-1 lines (Table II) may now be interpreted as a virtual absence of extracellular ATP, because there was a satisfactory response to stimulation of the endogenous adenylate cyclase.

To assess whether any leakage of other cytoplasmic components was present in the medium of Y-1 cells exposed to adenylate cyclase (21 nmol/min for 1 h at 37°C), we have measured the levels of some intracellular enzymes in the medium. No detectable amounts of glucose-6-phosphate dehydrogenase [27] and glutathione *S*-transferases [28] were found in the media of test dishes, as well as in the media of control dishes or in the media alone. The enzyme preparation itself contained measurable amounts of malate dehydrogenase [18], but none was found in the media of test dishes after the necessary correction, nor in the media of control dishes and in the media alone. Positive controls for all of these assays were provided by measurements of the intracellular enzymatic pools.

It seemed probable that ATP-consuming reactions such as hexokinase or creatine kinase might effectively compete with adenylate cyclase for cAMP generation in the media. The inability of these enzymes to enter cells readily would again confirm the extracellular nature of the reactions described here. As is apparent from Table IV, a hexokinase system effectively abolished cAMP generation in Y-1 cells. Basal cAMP levels were not significantly affected by hexokinase nor were the cellular levels of cAMP (data not shown). The medium ATP content showed the expected reciprocal relationship being reduced to below 10% by addition of adenylate cyclase and being undetectable after hexokinase (or serum) treatment. Similar results on cAMP generation were obtained with hexokinase treatment of CHO cells (Table IV), and with creatine kinase-dependent ATP consumption in Y-1 cells, although the latter enzyme

TABLE IV

EFFECT OF HEXOKINASE ON cAMP AND ATP CONCENTRATIONS

Incubation conditions were as follows: condition A, glucose (10 mM) plus Mg^{2+} (2 mM); condition B, glucose (10 mM) plus Mg^{2+} (2 mM) plus hexokinase (300 U/dish). DEAE-purified adenylate cyclase was incubated for 1 h at 37 °C at a concentration of 2.2 or 3.5 nmol/min for Y-1 or CHO cells respectively.

Cell type	Incubation conditions [a]	pmol cAMP/dish		pmol ATP/dish	
		control	DE	control	DE
Y-1	No additions	4.7	164	197	16
	condition A	4.6	281	257	11
	condition B	10	11	<1 [a]	<1
CHO	no additions	4.8	99	–	–
	condition A	4.1	140	–	–
	condition B	13	13	–	–

[a] Serum (12.5%) reduced ATP to less than 1 pmol/dish.

reduced cAMP concentrations by an average 85% under our conditions (data not shown).

Finally, the presence of serum virtually abolished the generation of extracellular cAMP by adenylate cyclase in both of these cell lines. This is shown in Table V. When 10% fetal calf serum was added to MEM, there was a 96% reduction in the yield of media cAMP. This was not merely due to the presence of protein, since BSA (3%) had no such effect and, indeed, produced some increase in the yield of cAMP. Similar effects could be observed when Y-1 cells were used. At a low cell density, the inhibitory effect of serum was greater than at a higher density (Table V). This suggested to us that some factor in the serum competed with the adenylate cyclase for its substrate, ATP, and this was probably an ATPase. Luciferase assays yielded linear dose-response curves to ATP added to MEM or F-10 medium. No ATP could be recovered in the presence of the amounts of horse serum or fetal calf serum normally used for growth media. Heat treatment at 56 °C restored recovery of ATP, suggesting that ATPase activity was the probable cause of the serum effect.

TABLE V

EFFECT OF SERUM ON cAMP GENERATION

21.4 nmol/min DEAE-adenylate cyclase activity (DE) was incubated for 1 h at 37 °C. The results are expressed as pmol cAMP/dish. FCS, fetal calf serum.

Cell type	Incubation conditions	Control	DE	% Inhibition
CHO	MEM	6.8	248	96.0
	MEM+10% FCS	6.0	16	
	MEM+3% BSA	7.5	390	
Y-1 (0.84 mg protein dish)	F-10	8.4	270	98.7
	F-10+12.5% serum	4.3	7.7	
Y-1 (1.38 mg protein dish)	F-10	15	642	76.6
	F-10+12.5% serum	20	167.	

Discussion

It is clear from the present results that leaked cellular ATP is the substrate for DE adenylate cyclase in the medium and the source of cAMP in that pool. Addition of the *Bordetella* adenylate cyclase preparation is not required, since control cells leak substantial amounts of ATP. In fact, ATP leaks from a number of tissues in situ and in the case of certain synapses acts as a neurotransmitter for purinergic nerves [20–24]. However, these experiments in no way rule out the possibility that *B. pertussis* extracts enhance such leakage, since they are known to contain proteins such as porin that could alter permeability [30]. In addition, ATP is extensively metabolized by a cascade of enzymes present on the cell surface [22,25,26]. Various stimuli enhance ATP release, and it is possible that a change of the culture medium is such a stimulus in CHO and Y-1 cells (Table III). Proteins are generally retained in the cell that leaks ATP, and we found that several enzymes did not leak from the cytoplasm. However, activation of the extracellular adenylate cyclase occurs via leaked calmodulin from the cells. This is seen with a variety of cells [7], and can be measured in conditioned media (unpublished observations). The extent of such stimula-

tion is likely to be submaximal, but is sufficient to permit ready demonstration of extracellular ATP. Finally, it is important to emphasize that no substantial fraction of the externally generated cAMP gains access to the cell interior (unpublished observation). The non-invasive form of the *Bordetella* adenylate cyclase is particularly useful in pointing this out.

The possibility, that the leaked ATP might permeabilize the cells used here, had to be considered in view of the abundant evidence that externally added ATP can increase the permeability of cells toward small molecules [21,23,25,31,32]. Although Trams [25] observed effects in astrocytes with 0.5 μM ATP, and 2 μM was effective in Ehrlich ascites cells [26], high concentrations (above 100 μM) are generally required to induce permeability changes. Assuming that half of the ATP in our experiments was converted to cAMP and that there was no destruction, the calculated maximum concentrations attained in these experiments is of the order of 0.1 μM, and the ATP concentrations accumulating under the conditions used here are probably insufficient to produce such effects. While the bulk of the catalytic activity of crude extracts of *B. pertussis* does not enter cells, studies from a number of laboratories have shown that there are several forms of the adenylate cyclase, and that there is a small fraction that penetrates a variety of cells and produces vast amounts of cAMP there [5,6,8–11]. Some of this cAMP can leak into the media from certain cells. This is not, however, the source of the extracellular cAMP in the present study, as we used a nonpenetrating form of the adenylate cyclase.

The present results do point out that the common practice of interpreting intracellular events from medium or total (cellular plus medium) cAMP levels after various forms of stimulation [15,19] can lead to erroneous conclusions when there is a chance of cAMP formation outside the cell. This may occur when there are broken cells but is marked when an exogenous adenylate cyclase is present. Moreover, even brief contact of residual external enzyme with intracellular ATP during processing creates enough cAMP to produce serious errors unless special precautions are taken to arrest catalysis by very active enzymes such as the *Bordetella* adenylate cyclase.

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References

1 Wolff, J., Cook, G.H., Goldhammer, A.R. and Berkowitz, S.A. (1980) Proc. Natl. Acad. Sci USA 77, 3841–3844.
2 Hewlett, E.L., Urban, M.A., Manclark, C.R. and Wolff, J. (1976) Proc. Natl. Acad. Sci. USA 73, 1926–1930.
3 Hewlett, E.L. and Wolff, J. (1976) J. Bacteriol. 127, 890–898.
4 Wolff, J., Cook, G.H., Goldhammer, A.R., Londos, C. and Hewlett, E.L. (1984) Adv. Cycl. Nucl. Res. 17, 161–172.
5 Confer, D.L. and Eaton, J.W. (1982) Science 217, 948–950.
6 Hanski, E. and Farfel, Z. (1985) J. Biol. Chem. 260, 5526–5532.
7 Goldhammer, A.R. and Wolff, J. (1982) Anal. Biochem. 124, 45–52.
8 Kessin, R.H. and Franke, J. (1986) J. Bacteriol. 166, 290–296.
9 Weiss, A.A., Hewlett, E.L., Myers, G.A. and Falkow, S. (1984) J. Infect. Dis. 150, 219–222.
10 Weiss, A.A., Hewlett, E.L., Myers, G.A. and Falkow, S. (1983) Infect. Immun. 42, 33–41.
11 Weiss, A.A., Myers, G.A., Crane, J.K. and Hewlett, E.L. (1986) in Microbiology (Leive, L., ed.), American Society for Microbiology, Washington, D.C.
12 Salomon, Y., Londos, C. and Rodbell, M. (1974) Anal. Biochem. 58, 541–548.
13 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
14 Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) Anal. Biochem. 150, 76–85.
15 Rae, P.A., Gutmann, N.S., Tsao, J. and Schimmer, B.P. (1979) Proc. Natl. Acad. Sci. USA 76, 1896–1900.
16 Koren, H.S., Anderson, S.J. and Larrick, J.W. (1979) Nature 279, 328–330.
17 Tsuchiye, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T. and Tada, K. (1980) Int. J .Cancer 26, 171–176.
18 Brooker, G., Harper, J.F., Terasaki, W.L. and Moylan, R.D. (1979) Adv. Cyclic Nucleotide Res. 10, 1–33.
19 Schimmer, B.P. and Zimmerman, A.E. (1976) Mol. Cell. Endocrinol. 4, 267–270.
20 Boyd, I.A. and Forrester, T. (1968) J .Physiol. 199, 115–135.
21 Rozengurt, E., Heppel, L.A. and Friedberg, I. (1977) J. Biol. Chem. 252, 4584–4590.
22 Pearson, J.D. and Gordon, J.L. (1979) Nature 281, 384–386.
23 Chaudry, I.H. (1982) Yale J. Biol. Med. 55, 1–10.

24 Burnstock, G. (1979) In Physiological and Regulatory Functions of Adenosine and Adenine Nucleotides, (Baer, H.P. and Drummond, G.I., eds.), pp. 3|132, Raven Press, New York.
25 Trams, E.G. (1974) Nature 252, 480–482.
26 Dubyak, G.R. and De Young, M.B. (1985) J. Biol. Chem. 260, 10653–10660.
27 De Moss, R.D. (1955) Methods Enzymol. 1, 328–334.
28 Habig, W.H. and Jakoby, W.B. (1981) Methods Enzymol. 77, 398–405.
29 Ochoa, S. (1955) Methods Enzymol. 1, 735–739.
30 Armstrong, L.K., Parr, T.R., Jr., Parker, C.D. and Hancock, R. (1986) J. Bacteriol. 166, 212–216.
31 Cockcroft, S. and Gomperts, B.D. (1979) Nature 279, 541–542.
32 Kitagawa, T. and Akamatsu, Y. (1986) Biochim. Biophys. Acta 860, 185–193.