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The invasive adenylate cyclase of *Bordetella pertussis*

Properties and penetration kinetics

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Bordetella pertussis, the causative organism of whooping cough, produces a calmodulin-sensitive adenylate cyclase. Confer & Eaton [(1982) Science **217**, 948–950] have shown that an extract from *B. pertussis* increases intracellular cyclic AMP levels in neutrophils and suggested that this increase is caused by the bacterial adenylate cyclase which penetrates these cells. We demonstrate in the present study that adenylate cyclase activity in lysates from lymphocytes exposed to a partially purified preparation of the bacterial enzyme has properties completely different from those of the intrinsic membrane-bound enzyme. Adenylate cyclase activity in lysates from lymphocytes exposed to the invasive enzyme is insensitive to *N*-ethylmaleimide, readily inactivated by acetic anhydride and relatively stable to SDS. Similar properties are exhibited by the bacterial enzyme itself. By contrast, the intrinsic membrane-bound enzyme activated by forskolin and guanosine 5′-γ-thiotriphosphate is sensitive to *N*-ethylmaleimide and SDS and relatively stable to acetic anhydride. This strongly supports the notion that *B. pertussis* adenylate cyclase penetrates cells. Using the partially purified preparation of the invasive enzyme, we have studied the kinetics of its penetration. The intracellular catalytic activity reaches a steady state within 20 min, irrespective of enzyme or cell concentration. Steady-state levels are maintained for at least 2 h provided that the invasive enzyme is present in the incubation medium. Upon its removal, a rapid decrease ($t_{\frac{1}{2}} \approx 15$ min) in the intracellular cyclase level is observed. This decrease reflects intracellular inactivation of the bacterial enzyme and is not caused by the release of the enzyme to the cell medium.

INTRODUCTION

Bordetella pertussis, the causative organism of whooping cough, produces an adenylate cyclase which has several unique features. Its location in the organism is predominantly extracytoplasmic (Hewlett *et al.*, 1976), and it is highly responsive to calmodulin (CaM), which is not found in the organism (Wolff *et al.*, 1980). In contrast with the eukaryotic membrane-bound enzyme, the bacterial adenylate cyclase is unresponsive to hormones, guanine nucleotides and forskolin (Wolff *et al.*, 1983). Confer & Eaton (1982) have shown that exposure of human neutrophils to a dialysed urea extract of *B. pertussis* leads to a rapid and extensive production of intracellular cyclic AMP, with consequent paralysis of their phagocytic functions. Confer *et al.* (1984) have presented preliminary evidence suggesting that this unregulated generation of cyclic AMP is caused by internalization of the bacterial adenylate cyclase rather than by stimulation of the intrinsic membrane-bound enzyme. Since then, several laboratories, using different approaches, provided evidence supporting this notion. Weiss *et al.* (1985) have shown that *B. pertussis* mutants deficient of adenylate cyclase activity are avirulent, and extract prepared from these mutants does not raise cyclic AMP levels in S49 lymphoma cells. We have demonstrated selectivity in the response of various cells to *B. pertussis* extract, and a time- and temperature-dependent accumulation of catalytic activity in lymphocytes (Hanski & Farfel, 1985). Shattuck & Storm (1985) have shown that addition of CaM to a preparation of partially purified *B. pertussis* adenylate cyclase devoid of islet-activating protein prevents cyclic AMP increase in neuroblastoma and human red blood cells.

In the present study we investigate the properties of adenylate cyclase in lysates from lymphocytes exposed to a partially purified preparation of the bacterial enzyme. The cyclase activity in this lysate has different sensitivities to various reagents compared with the membrane-bound enzyme, thus further establishing the notion that *B. pertussis* adenylate cyclase penetrates cells. In addition, we have further characterized the penetration kinetics of this invasive enzyme to lymphocytes.

MATERIALS AND METHODS

Materials

[α-^{32}P]ATP (80 Ci/mmol) and cyclic [^{3}H]AMP (27 Ci/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. ATP (A-2383), phosphoenolpyruvate, pyruvate kinase, SBTI, acetylated trypsin, EGTA, PMSF and NEM were obtained from Sigma. Ultrogel AcA 34 was obtained from LKB, and Dowex AG 50W-X4 resin was obtained from Bio-Rad Laboratories. GTPγS was purchased from Boehringer-Mannheim and forskolin from Calbiochem. Acetic

Abbreviations used: PBS, phosphate-buffered saline (5 mM-sodium phosphate/140 mM-NaCl, pH 7.4); PMSF, phenylmethanesulphonyl fluoride; CHO, Chinese-hamster ovary; GTPγS, guanosine 5′-[γ-thio]triphosphate; NEM, *N*-ethylmaleimide; DTT, dithiothreitol; CaM, calmodulin; LDH, lactate dehydrogenase; SBTI, soybean trypsin inhibitor; FIC, fractions inducing cyclic AMP accumulation; IAP, islet-activating protein.

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anhydride and SDS were purchased from BDH Chemicals. All other reagents were of the finest available grade from commercial sources.

Methods

Culture of organism and preparation of partially purified enzyme. *B. pertussis* strain 165 was cultured in modified Stainer–Scholte medium as detailed by Hewlett & Wolff (1976). Some of the bacterial preparations used were kindly supplied by Dr. J. Shiloach of the National Institutes of Health, Bethesda, MA, U.S.A., and had identical properties. A dialysed urea extract of *B. pertussis* was prepared essentially by the procedure of Confer & Eaton (1982), except for the following modifications: the extraction solution contained 5.3 M-urea and 10 μM-PMSF. The extract was dialysed against a solution containing 10 mM-Hepes, 100 mM-NaCl, 10 μM-PMSF and 5 mM-2-mercaptoethanol, pH 7.4. The organism and the cell-free supernatant were separated by centrifugation at 35000 rev./min for 60 min in Beckman 35 fixed-angle rotor (143000 $g_{max.}$). The supernatant obtained was subjected to gel filtration on an Ultrogel ACA 34 column according to the procedure described by us previously (Hanski & Farfel, 1985). The peak of fractions inducing cyclic AMP accumulation (FIC) in human lymphocytes (see Fig. 3*a* of Hanski & Farfel, 1985) was pooled and concentrated 10-fold on Amicon PM 10 filter. The protein concentration of various FIC preparations ranged between 1 and 2 mg/ml. Concentrated FIC was used as a source of the invasive enzyme in the studies presented below.

Human lymphocytes. Human lymphocytes were isolated from whole blood as described by us previously (Hanski & Farfel, 1985).

Determination of the invasive-enzyme activity. Cells [(1–3) $\times 10^6$] were incubated with FIC, usually in 0.5 ml of PBS containing 1 mM-$MgCl_2$ and 1 mM-$CaCl_2$ at 36 °C. At the end of the incubation periods cells were transferred to ice, then centrifuged (at 10000 *g*) for 2 min in Eppendorf centrifuge and supernatants were discarded. Removal of the non-penetrating enzyme that adheres to cell surface was performed by the trypsin treatment described by us previously (Hanski & Farfel, 1985). Briefly, cells were incubated with acetylated trypsin (400 μg/ml) at 10 °C. After 10 min, SBTI (800 μg/ml) was added and cells were washed twice at 4 °C as described above. Disruption of cell pellets was performed by the addition of 100 μl of HME buffer (20 mM-Hepes/2 mM-$MgCl_2$/1 mM-EDTA, pH 7.4) supplemented with 1 mM-DTT, 10 μM-PMSF and 0.1% Lubrol, followed by freezing and thawing twice in liquid N_2. Samples of lysates (10 μl each) were assayed for adenylate cyclase activity. For determination of the decay rate of adenylate cyclase activity and the appearance of catalytic activity in the medium, (1–3) $\times 10^7$ cells were preincubated with FIC usually in 5 ml of PBS at 36 °C. At the end of the incubation periods, cells were transferred to ice and washed twice by centrifugation at 1400 rev./min (*r* 20.0 cm) in an IEC centrifuge for 10 min at 4 °C. Washed cells were subjected to the trypsin treatment described in Hanski & Farfel (1985) and 0.5 ml aliquots were reincubated at 36 °C. Reincubation was stopped by transfer to ice and centrifugation for 2 min in an Eppendorf centrifuge. Supernatants were then separated from cell pellets. Pellets were disrupted as described above and three samples of 10 μl each were assayed for adenylate cyclase activity. For determination of adenylate cyclase activity in the supernatant, three samples of 30 μl each were used.

Adenylate cyclase assay. Adenylate cyclase activity determinations were performed as described by us previously (Hanski & Farfel, 1985). All results are presented as means for triplicate assays, the S.E.M. being less than 5%.

Cyclic AMP determination. Intracellular cyclic AMP was determined as described by us previously (Hanski & Farfel, 1985). For determination of total cyclic AMP, (1–3) $\times 10^6$ cells were incubated in 0.5 ml of PBS at 36 °C with FIC. At the end of incubation periods cell suspensions were transferred to ice and 0.5 ml of 5% (w/v) $HClO_4$ was added. Cells were left on ice for 15–30 min, then centrifuged at 10000 *g* for 10 min in an Eppendorf centrifuge. Samples (800 μl) of the supernatant were withdrawn and the pH was raised to 7.0 by the addition of 80 μl of a factorized solution of KOH. An internal standard ($\approx$ 10000 c.p.m.) of cyclic [^{32}P]AMP (sp. radioactivity 40000 c.p.m./pmol) was added and the final volume was adjusted to 1 ml by addition of water. The samples were applied to 1 ml Dowex AG 50W-X4 (200–400 mesh) resin columns. The columns were washed with 2 ml of water and then eluted with 4 ml of water. The collected effluents were freeze-dried and reconstituted by addition of 0.5 ml of 50 mM-sodium acetate, pH 4.0. Three samples of 50–100 μl of the reconstituted solution were used for cyclic AMP determination. The standard cyclic [^{32}P]AMP was produced by incubation of [α-^{32}P]ATP (0.1 mM, 40000 c.p.m./pmol) with an excess of dialysed urea extract under conditions similar to those described for the assay of adenylate cyclase activity. Cyclic [^{32}P]AMP was separated from unchanged [α-^{32}P]-ATP (which was less than 1%) by sequential column chromatography over Dowex AG 50W-X4 resin and neutral alumina. All results are presented as means for triplicate assays, the S.E.M. being less than 10%.

LDH. LDH activity was determined as described by Decker (1977).

Protein. Protein was determined by the method of Bradford (1976).

RESULTS

Properties of partially purified *B. pertussis* invasive adenylate cyclase

We have previously reported (Hanski & Farfel, 1985) that incubation of human lymphocytes with a dialysed urea extract of *B. pertussis* at 4 °C followed by washing of cells does not lead to cyclic AMP accumulation, even if the cells are subsequently incubated at 36 °C. Under these conditions, and in spite of extensive repeated washings, some cyclase activity remains adsorbed to the cell surface. Most of the adhered cyclase activity can be removed by the trypsin treatment decribed by us previously, thus allowing determination of the intracellular cyclase activity (Hanski & Farfel, 1985). Gel filtration of dialysed urea extract on an Ultrogel ACA 34 column shows that the peak of FIC in lymphocytes

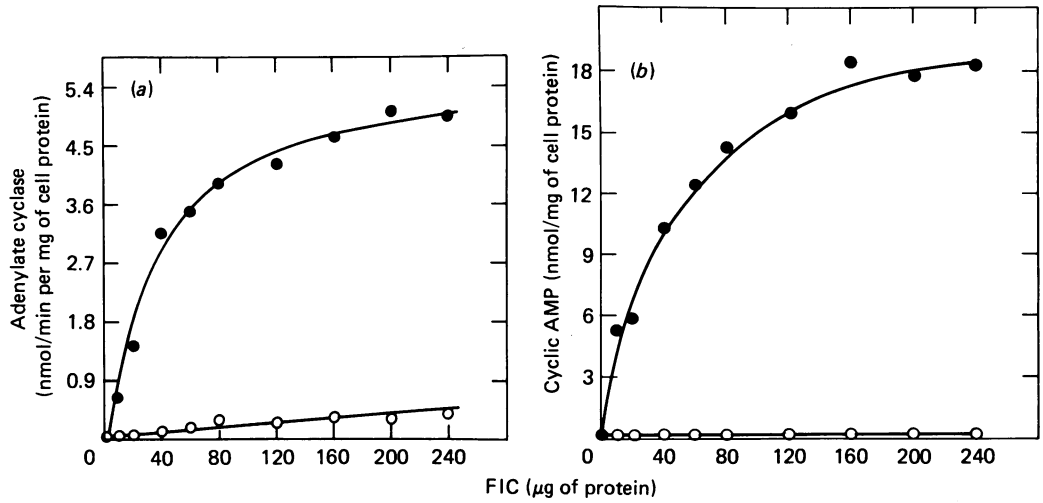

Fig. 1. Dependence of intracellular adenylate cyclase activity accumulation and cyclic AMP generation on FIC concentration

Human lymphocytes (1.5×10^6 cells) were incubated at 36 °C (●) or at 4 °C (○) with the indicated quantities of FIC for 60 min in a final volume of 0.75 ml. Intracellular catalytic activity (*a*) and total cyclic AMP (*b*) were determined as described under 'Methods'.

migrates between a 'heavy' and a 'light' peak of adenylate cyclase activity and constitutes less than 10% of total cyclase activity found in the extract (Hanski & Farfel, 1985).

We concentrated FIC 10-fold and used it as a source of the invasive enzyme. The specific activity of various FIC preparations ranged from 0.5 to 1 μmol/min per mg of protein, and its protein concentration varied from 1 to 2 mg/ml. No IAP presence was detected in these preparations. This was examined by using [^{32}P]NAD for labelling of the inhibitory guanine-nucleotide-binding protein G_i in S49 cyc$^-$ lymphoma cell membranes (results not shown).

Incubation of human lymphocytes with FIC at 36 °C resulted in the accumulation of high levels of both catalytic activity and cyclic AMP in these cells. Figs. 1(*a*) and 1(*b*) show the accumulation of cyclase activity and cyclic AMP respectively in lymphocytes as a function of FIC concentration. Both activities demonstrated similar saturable dose–response curves. The amount of cyclase activity that remained adhered to the cell surface after trypsin treatment was assessed by incubation of cells with increasing concentrations of FIC at 4 °C. As shown in Fig. 1(*a*), this activity constitutes less than 10% of the total activity at saturation. The amounts of catalytic activity accumulated and cyclic AMP generated were both dependent on FIC and cell concentration; at higher cell concentrations saturation of catalytic activity and cyclic AMP generation were reached at lower FIC concentrations (results not shown).

Evidence for penetration of the invasive enzyme into lymphocytes

Since pure preparations of *B. pertussis* invasive adenylate cyclase are currently not available, the possibility that the increase in cyclic AMP and cyclase activity is caused by activation of the membrane-bound enzyme rather than by penetration of the invasive bacterial enzyme was tested. Fig. 2 demonstrates that the properties of adenylate cyclase in lysates from lymphocytes exposed to the invasive enzyme were completely different from those of the intrinsic membrane-bound enzyme, with respect to specific activity and sensitivity to NEM, acetic anhydride and SDS. The specific activity of cells exposed to half-maximal concentration of the invasive enzyme (Fig. 1*a*) was about 2 nmol/min per mg of cell protein. Lysates prepared from lymphocytes and activated by forskolin and GTPγS had a specific activity which was 20-fold lower. Adenylate cyclase activity measured in FIC was insensitive to NEM, but was readily inactivated by acetic anhydride (Fig. 2*a*). Similar sensitivity to acetic anhydride (Fig. 2*c*) and resistance to NEM (Fig. 2*b*) were observed in lysates from lymphocytes exposed to the bacterial enzyme. In contrast, the membrane-bound enzyme activated by GTPγS and forskolin was sensitive to NEM (Fig. 2*b*), but was relatively stable to acetic anhydride (Fig. 2*c*). Furthermore, cyclase activity in lymphocytes exposed to the bacterial enzyme was more stable to SDS in comparison with the membrane-bound enzyme (Fig. 2*d*), even though the latter was in its pre-activated, more stable, form (Smigel, 1986).

Characterization of penetration kinetics of *B. pertussis* adenylate cyclase to lymphocytes

Time courses of cyclase activity and cyclic AMP accumulation, at three different FIC concentrations, are shown in Figs. 3(*a*) and 3(*b*) respectively. Both activities exhibited a similar kinetic behaviour; for each concentration of FIC used, a plateau was reached within 15–20 min and maintained for 3 h. The cells were viable for 4 h of incubation at 36 °C as judged by Trypan Blue exclusion and by the absence of LDH activity in the cell medium. The time courses of cyclase activity or cyclic AMP accumulation were independent of either FIC or cell concentration (results not shown). However, the plateau levels obtained were proportional to the concentrations of FIC used (Figs. 3*a* and 3*b*). In order to examine the possibility that the plateau level of intracellular cyclase activity represents a dynamic process in which there is a continuous penetration and inactivation, we have performed the experiment shown in Fig. 4. Cells were preincubated at 36 °C with FIC for 6 min and the incubation medium was removed, as was adsorbed

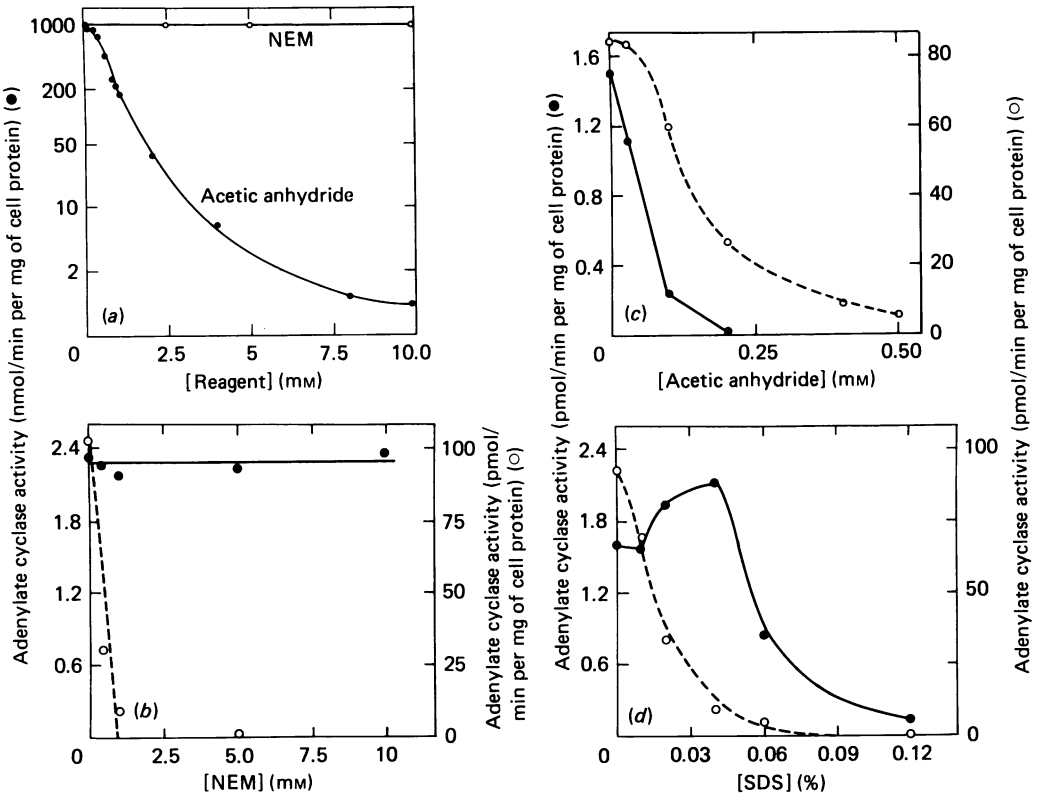

Fig. 2. Differential susceptibility to various reagents of (1) adenylate cyclase of partially purified bacterial enzyme (FIC), (2) lymphocyte intrinsic membrane-bound adenylate cyclase and (3) adenylate cyclase activity of lysates of lymphocytes exposed to partially purified *B. pertussis* invasive enzyme (preloaded lysates)

(*a*) Effect of NEM and acetic anhydride on *B. pertussis* adenylate cyclase. To remove mercaptoethanol, FIC was extensively dialysed against buffer containing 10 mM-Hepes, 100 mM-NaCl and 1 mM-$CaCl_2$ at pH 7.4. Samples (15 μl) of dialysed FIC were incubated at 20 °C with 15 μl samples of NEM at the final indicated concentrations. After 5 min, 10 μl samples of DTT, at concentrations which produced 1 mM excess of DTT over the corresponding NEM concentration used, were added. The mixture was further incubated for 5 min and then incubated at 4 °C. Two aliquots of 10 μl each were withdrawn, diluted 200-fold into HME buffer and assayed as described under 'Methods'. Acetic anhydride treatment was performed essentially as described above; dialysed FIC samples were incubated with the indicated final concentrations of acetic anhydride at 4 °C for 1 h. Excess of acetic anhydride was quenched by the addition of 6-aminohexanoic acid to a final concentration of 25 mM. After 1 h incubation, samples were diluted and assayed as described above. (*b*) Differential effect of NEM on the intrinsic membrane-bound adenylate cyclase and adenylate cyclase in preloaded lymphocytes. Lymphocytes (2×10^7 cells) were incubated with 0.5 mg of FIC at 36 °C in 5 ml of PBS. Control cells were incubated under identical conditions but in the absence of FIC. After 60 min, cells were transferred to ice, washed, trypsin-treated and disrupted in final volume of 0.5 ml of HME as described under 'Methods'. The lysate obtained from control cells was incubated for 15 min at 36 °C, in the presence of 0.1 mM-GTPγS, 0.1 mM-forskolin and 10 mM-$MgCl_2$, in order to activate and stabilize the membrane-bound enzyme. NEM treatment was conducted as described in (*a*). Adenylate cyclase activity in lysates from control cells was determined in triplicate in a standard reaction mixture containing 0.1 mM-GTPγS and 0.1 mM-forskolin (○). Adenylate cyclase activity in preloaded lymphocyte lysate was determined in triplicate, in an assay mixture containing CaM and Ca^{2+} (●) as described under 'Methods'. (*c*) Differential effect of acetic anhydride on the intrinsic membrane-bound adenylate cyclase and adenylate cyclase in preloaded lymphocytes. Lysates from lymphocytes incubated with (●) or without (○) FIC were obtained and treated as described in (*b*). Acetic anhydride treatment was conducted as described in (*a*). (*d*) Differential effect of SDS on the intrinsic membrane-bound adenylate cyclase and adenylate cyclase in preloaded lymphoctes. Lysates from lymphocytes incubated with (●) or without (○) FIC were obtained and treated as described in (*b*). Lysate samples (20 μl, containing 40 μg of protein) were incubated at 4 °C with 20 μl of SDS at the final indicated concentrations. Adenylate cyclase activities were determined as described in (*b*).

cyclase activity (see under 'Methods'). Cells were then reincubated at 36 °C and, at the indicated times, cells were disrupted and the amount of intracellular cyclase activity determined. As shown in Fig. 4, removal of extracellular adenylate cyclase led to a decrease in the intracellular cyclase activity. This decay was dependent on cell integrity; there was no decay of cyclase activity in disrupted cells. The cyclase activity in FIC was also completely stable when incubated in the presence of cell lysates at 36 °C (results not shown). To examine whether the observed decrease in the activity of the intracellular enzyme represents inactivation process or a release of the enzyme into cell medium, we have performed the experiment shown in Fig. 5. Cells were incubated with the invasive enzyme for 6 min and then treated as described in Fig. 4. A small amount of catalytic activity appeared in the medium after a lag time of 15 min and reached its maximal level within 60 min. This maximal

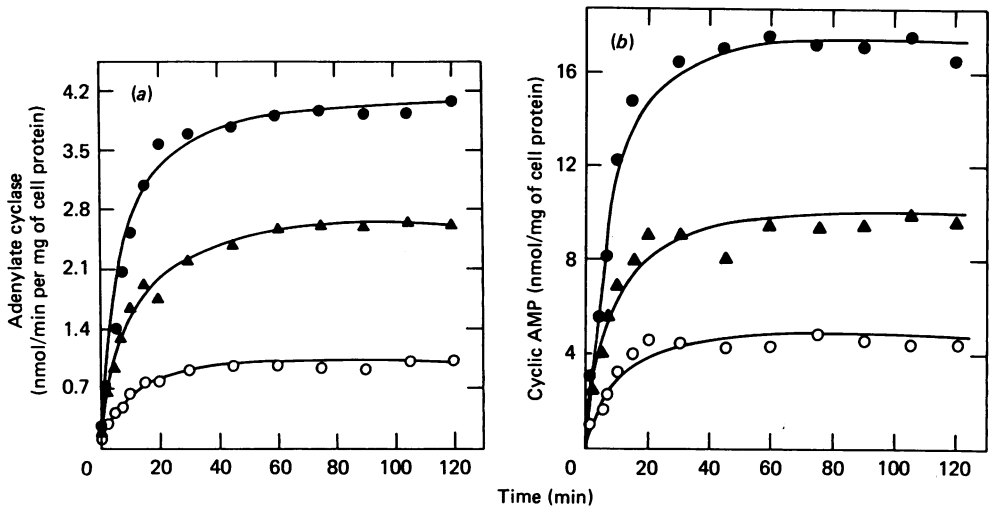

Fig. 3. Time course of intracellular adenylate cyclase activity accumulation and cyclic AMP generation

Lymphocytes (1.5×10^6 cells) were incubated in 0.5 ml (final vol.) at 36 °C with increasing quantities of FIC: 16 μg (○), 32 μg (▲) and 100 μg (●). At the indicated times, intracellular cyclase activity (*a*) and total cyclic AMP (*b*) were determined as described under 'Methods'.

level represented only about 6% of the maximal intracellular catalytic activity (Fig. 4). The release of enzyme activity to the medium was not the result of loss of cell integrity, since no appreciable amounts of cyclic AMP or LDH activity were detected in the medium (results not shown). Furthermore, this release required the presence of extracellular Ca^{2+} and was blocked by EGTA (Fig. 5), similar to the Ca^{2+} requirement for penetration [Fig. 5 (inset) and Hanski & Farfel (1985)].

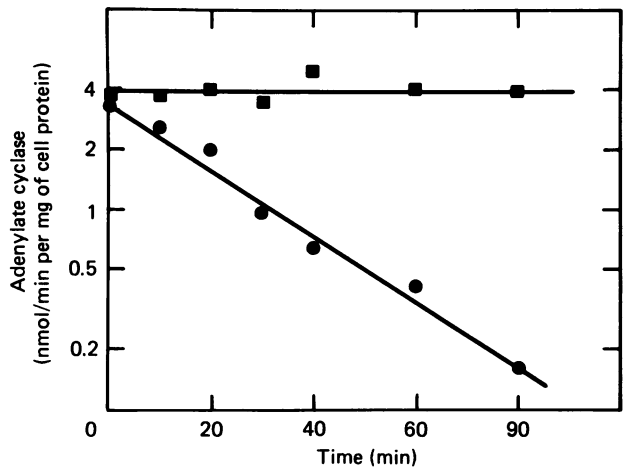

Fig. 4. Intracellular decrease of adenylate cyclase activity

Lymphocytes (4×10^7 cells) were incubated in PBS with 1 mg of FIC. After 6 min, cells were transferred to 4 °C, washed and trypsin-treated as described under 'Methods'. Treated cells were resuspended in 8 ml of PBS and divided into two equal pools; one pool was reincubated at 36 °C. At the indicated times, 0.5 ml samples were withdrawn, transferred to ice and intracellular cyclase activity was determined in triplicate (●), as described under 'Methods'. The second pool was centrifuged and disrupted in 0.4 ml of HME as described under 'Methods'. The lysate obtained was reincubated at 36 °C and, at the indicated times, three samples of 10 μl each were withdrawn and cyclase activity (■) was determined.

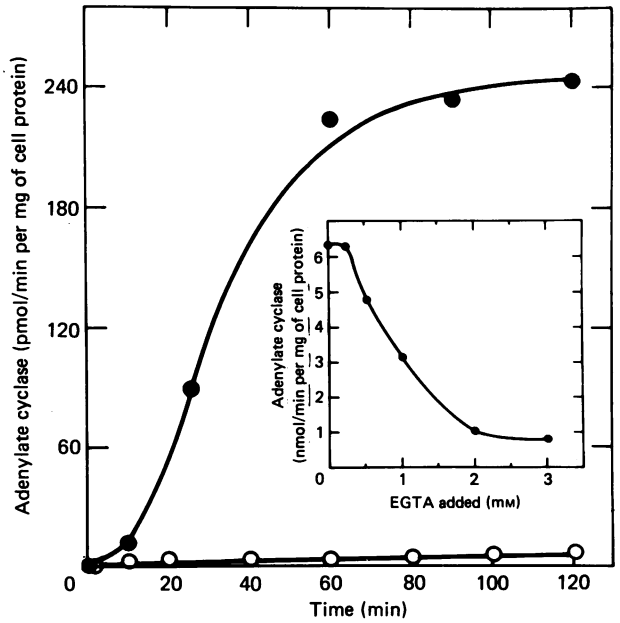

Fig. 5. Appearance of adenylate cyclase activity in the medium

Lymphocytes (4×10^7 cells) were incubated for 6 min in 5 ml of PBS with 0.5 mg of FIC, then washed and trypsin-treated as described under 'Methods'. Treated cells were resuspended in 4 ml of PBS containing 1 mM-Ca^{2+} (●) or in 5 ml of PBS containing 1 mM-Ca^{2+} and 2 mM-EGTA (○). Both pools were reincubated at 36 °C. At the times indicated, aliquots (0.5 ml each) were withdrawn, transferred to ice and supernatants were separated from cell pellets as described under 'Methods'. $CaCl_2$ was added to produce 1 mM excess over EGTA and 30 μl samples from each supernatant were assayed in triplicate for adenylate cyclase activity as described under 'Methods'. Inset: EGTA blocks adenylate cyclase accumulation in cells. Lymphocytes (2×10^6 cells) were incubated with 40 μg of FIC in 0.5 ml of PBS in the presence of the indicated concentrations of EGTA. After a 30 min incubation at 36 °C, cells were incubated at 4 °C, washed, trypsin-treated, and the intracellular catalytic activity was determined as described under 'Methods'.

DISCUSSION

Confer & Eaton (1982) were the first to suggest that the brisk increase in cyclic AMP observed when neutrophils are exposed to dialysed urea extract from *B. pertussis* is caused by penetration of the bacterial adenylate cyclase. Since then, several laboratories using different approaches have provided evidence supporting this notion. Studies using a series of transposon-Tn5-induced mutants of *B. pertussis* indicated that the bacterial adenylate cyclase rather than IAP was responsible for the increase of cyclic AMP level observed in S49 lymphoma cells (Weiss *et al.*, 1985). Furthermore, mutants deficient in adenylate cyclase were severely impaired in their ability to produce whooping cough in an animal model of the disease. Activation of the membrane-bound adenylate cyclase in S49 lymphoma cells through the β-adrenergic receptor, G_s, and the catalyst by isoprenaline, cholera toxin and forskolin respectively was inhibited by somatostatin. In contrast, somatostatin had no effect on cyclic AMP accumulation in S49 lymphoma cells exposed to *B. pertussis* extract containing the bacterial enzyme (Hewlett *et al.*, 1985). We have demonstrated cell selectivity in the response of various cells to *B. pertussis* extract, and a time- and temperature-dependent accumulation of catalytic activity in lymphocytes exposed to the bacterial enzyme (Hanski & Farfel, 1985). Shattuck & Storm (1985), demonstrated increased cyclic AMP levels in neuroblastoma and human red blood cells when using a partially purified enzyme from *B. pertussis* culture medium devoid of IAP activity. Addition of CaM to this enzyme preparation blocked its ability to cause cyclic AMP accumulation in the cells, suggesting that the bacterial enzyme is active within the cell. To support further the notion that *B. pertussis* adenylate cyclase penetrates cells, we have characterized the properties of the enzyme in lysates from lymphocytes exposed to the bacterial enzyme (preloaded lysates). The properties of this cyclase activity were similar to those of the bacterial cyclase, but completely different from those of the intrinsic membrane-bound enzyme with respect to sensitivity to NEM, acetic anhydride and SDS. The specific activity of adenylate cyclase in preloaded lysates was 40–80-fold higher than that of the fully activated intrinsic membrane-bound enzyme.

Only about 2% of total catalytic activity present in FIC gained access to lymphocytes, as calculated from the linear portion of Fig. 1(*a*). However, the plateau levels of the intracellular catalytic activity obtained in lymphocytes exposed to different concentrations of FIC (Fig. 3*a*) were directly proportional to the amount of FIC used. Thus it seems that catalyst concentration is not the rate-limiting factor and another factor is required to facilitate penetration. The concentration of this 'inserting factor' probably determines the amount of catalyst penetrated. A precedent for the existence of such factor was demonstrated for anthrax toxin. This toxin is composed of three polypeptides, oedema factor, which is a soluble CaM-dependent adenylate cyclase, lethal factor and a protective antigen (Leppla, 1982). Leppla (1982) suggested that the protective antigen binds to cell-surface receptors and facilitates penetration of a stoichiometric amount of oedema factor to CHO cells.

Cholera toxin (Moss & Vaughan, 1979), pertussis toxin (Katada & Ui, 1980) and other bacterial toxins exert their biological effects after various lag periods, the durations of which are dependent on toxin concentration and cell type. In contrast, the accumulation of *B. pertussis* cyclase activity and cyclic AMP proceeded without a lag (Figs. 3*a* and 3*b*); plateau levels were reached within 15–20 min and the rates of cyclase and cyclic AMP accumulation were independent of FIC or cell concentration. The time course of cyclase and cyclic AMP accumulation is slightly more rapid than that reported by us previously (Hanski & Farfel, 1985). This probably reflects the use of a purer preparation as a source of the invasive enzyme. The fact that enzyme's entry is rapid and proceeds without a lag period suggests that it occurs by direct penetration of the plasma membrane. Since cyclic AMP generation parallels intracellular cyclase accumulation, the activation of the bacterial enzyme must also be rapid, occurring coincidentally with enzyme's entry event.

Removal of extracellular enzyme leads to a decrease in its intracellular activity, indicating continuous penetration of the enzyme. Therefore the plateau levels shown in Fig. 3(*a*) represent a steady state which is determined by penetration and decay rates of bacterial cyclase activity. The ability of lymphocytes to maintain these steady-state levels of intracellular cyclase activity for at least 2 h indicates that the cellular components involved in the uptake of the invasive enzyme are not consumed during enzyme action. Leppla (1982) observed a rapid decay in intracellular cyclic AMP level upon removal of oedema factor from culture medium of CHO cells. This rapid decay of cyclic AMP, apparently, reflects inactivation of oedema factor in CHO cells. The decrease of intracellular *B. pertussis* cyclase activity in lymphocytes cannot be explained by extrusion of the invasive enzyme, since only about 6% of the maximal level of the intracellular catalytic activity was detected in the medium. The appearance of enzyme's catalytic activity in the medium required extracellular Ca^{2+}, was temperature-dependent and was not caused by loss of cell integrity. Release of toxin into the medium was described for ricin by Sandvig & Olsnes (1979). Thus the observed decrease in adenylate cyclase represents an inactivation process which occurs within the cell and is dependent on cell integrity. In the accompanying paper (Farfel *et al.*, 1987) we demonstrate that the invasive enzyme is associated with a membrane fraction throughout the process of its penetration.

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REFERENCES

Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
Confer, D. L. & Eaton, J. W. (1982) Science **217**, 948–950
Confer, D. L., Slungaard, A. S., Graf, E., Panter, S. S. & Eaton, J. W. (1984) Adv. Cyclic Nucleotide Protein Phosphorylation Res. **17**, 183–187
Decker, L. A. (ed.) (1977) Worthington Enzyme Manual, Worthington Biochemical Corporation, Freehold, NJ
Farfel, Z., Friedman, E. & Hanski, E. (1987) Biochem. J. **243**, 153–158
Hanski, E., & Farfel, Z. (1985) J. Biol. Chem. **260**, 5526–5532
Hewlett, E. L. & Wolff, J. (1976) J. Bacteriol. **127**, 890–898

Hewlett, E. L., Urban, M. A. Manclark, C. R. & Wolff, J. (1976) Proc. Natl. Acad. Sci. U.S.A. **73**, 1926–1930
Hewlett, E. L., Weiss, A. A., Pearson, R. D., Myers, G. W. & Cronin, M. J. (1985) in Pertussis Toxin (Sekura, R. D., Moss, J. & Vaughan, M., eds.), pp. 241–250, Academic Press, New York
Katada, T. & Ui, M. (1980) J. Biol. Chem. **255**, 9580–9588
Leppla, S. H. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 3162–3166
Moss, J. & Vaughan, M. (1979) Annu. Rev. Biochem. **48**, 581–601
Sandvig, K. & Olsnes, S. (1979) Exp. Cell. Res. **121**, 15–25
Shattuck, R. L. & Storm, D. R. (1985) Biochemistry **24**, 6323–6328
Smigel, M. D. (1986) J. Biol. Chem. **261**, 1976–1982
Weiss, A. A., Hewlett, E. L., Mayers, G. A., & Falkow, S. (1985) J. Infect. Dis. **150**, 219–223
Wolff, J., Cook, G., Goldhammer, A., & Berkowitz, S. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 3841–3844
Wolff, J., Cook, G. H., Goldhammer, A. R., Londos, C. & Hewlett, E. L. (1984) Adv. Cyclic Nucleotide Res. **17**, 161–173

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