Biochem. J. (1988) 253, 735–743 (Printed in Great Britain)

Interactions of cholera toxin with isolated hepatocytes

Effects of low pH, chloroquine and monensin on toxin internalization, processing and action

Michel JANICOT, Jean-Pierre CLOT and Bernard DESBUQUOIS
Institut National de la Santé et de la Recherche Médicale, Unité 30, Hôpital des Enfants Malades, 75015 Paris, France

The major steps in cholera-toxin action, i.e. binding, internalization, generation of A_1 peptide and activation of adenylate cyclase, were examined in isolated hepatocytes. The binding of toxin involves a single class of high-affinity sites ($K_D \simeq 0.1$ nM; $B_{max.} \simeq 10^7$ sites/cell). At 37 °C, cell-associated toxin is progressively internalized, as judged by the loss of its accessibility to antibodies against whole toxin, A and B subunits (about 50, 75 and 30% of initially bound toxin after 40 min respectively). Two distinct pathways are involved in this process: endocytosis of the whole toxin, and selective penetration of the A subunit into the plasma membrane. Exposure of hepatocytes to an acidic medium (pH 5) results in a rapid and marked disappearance of the A subunit from the cell surface. Generation of A_1 peptide and activation of adenylate cyclase by the toxin occur after a lag phase (10 min at 37 °C), and increase with time in a parallel manner up to 2–3% A_1 peptide generated; they are unaffected by exposure of cells to an acidic medium. Chloroquine and monensin, which elevate the pH in acidic organelles, inhibit by 2–4-fold both the generation of A_1 peptide and the activation of adenylate cyclase. Unexpectedly, these drugs also inhibit the internalization of the toxin. These results suggest that an acidic pH facilitates the penetration of A subunit into the plasma membrane and presumably the endosomal membrane as well, and that endocytosis of cholera toxin is required for generation of A_1 peptide and activation of adenylate cyclase.

INTRODUCTION

Cholera toxin, a toxin secreted by the bacterium *Vibrio cholerae*, exerts its effects on mammalian cells by activation of adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1]. Structurally, the toxin is composed of two different subunits, A and B (Finkelstein, 1973; van Heyningen, 1976; Moss & Vaughan, 1979; Holgrem, 1981). The initial step in cholera-toxin action is the binding of this toxin through its B subunit to a specific cell-surface receptor, the monosialoganglioside GM_1 (Cuatrecasas, 1973*b*). After a lag phase, the A subunit is reduced, and peptide A_1 catalyses the activation of adenylate cyclase through the NAD-dependent ADP-ribosylation of the stimulatory component of the enzyme, G_S (Cassel & Pfeuffer, 1978; Gill & Meren, 1978; Moss *et al.*, 1979). It is generally accepted that, during this lag, cholera toxin or some part of it undergoes translocation across the plasma membrane. However, it has previously been shown that cholera toxin undergoes adsorptive endocytosis (Joseph *et al.*, 1978, 1979; Montesano *et al.*, 1982), and that, at least in liver, reduction of the A subunit occurs in the endosomal compartment (Janicot & Desbuquois, 1987). These findings raise the possibility that endocytosis of the toxin may be required for access of the A_1 peptide to the cytoplasmic domain of the plasma membrane and subsequent activation of adenylate cyclase.

In the present study, the functional relationships between the internalization of cholera toxin, the generation of the A_1 peptide and the activation of adenylate cyclase in isolated hepatocytes have been examined. As the internalized toxin encounters a low pH in the endosomal compartment, we have examined, in this cell system, whether: (1) lowering the pH of the medium allows and/or facilitates the translocation of the toxin (or some part of it) across the plasma membrane; and (2) increasing the endosomal pH by pharmacological means inhibits toxin processing and action.

MATERIALS AND METHODS

Cholera toxin and antitoxins

Cholera toxin was obtained from the Swiss Serum and Vaccine Institute (Bern, Switzerland); this preparation was used to prepare ^{125}I-labelled toxin. Cholera toxin was also kindly given by Dr J. L. Tayot and Dr J. R. Cartier (Institut Mérieux, Lyon, France). Rabbit antisera against whole cholera toxin (IgG-CT), cholera toxin A subunit (IgG-CT_A) and cholera toxin B subunit (IgG-CT_B) were kindly given by Dr P. Dodin (Institut Pasteur, Paris, France). The affinities of IgG-CT, IgG-CT_A and IgG-CT_B for native cholera toxin, similar for the three antisera, were about 10^8 M^{-1}, and their binding capacities were 280, 7 and 46 μg of native toxin/ml of undiluted serum respectively. The antiserum dilutions used in this work (selected so as to ensure maximal binding of free cholera toxin) were 1:200 for both IgG-CT and IgG-CT_B and 1:20 for IgG-CT_A. At these dilutions, 95%, 60% and 90% of ^{125}I-labelled toxin (50000 c.p.m.; 150 nmol) was bound to antibody respectively.

Chemicals

Carrier-free Na^{125}I, [α-^{32}P]ATP and cyclic [^{3}H]AMP were from Amersham International (Amersham, Bucks., U.K.). Bovine serum albumin (fraction V) was from Miles Laboratories. EDTA was from Merck. ATP, GTP, highly purified bovine serum albumin, monensin, EGTA and 3-isobutyl-1-methylxanthine were purchased from Sigma. Chloroquine sulphate was from Specia. Collagenase, phosphocreatine and creatine kinase were from

Boehringer. Protein A (*Staphylococcus aureus*) was from Pharmacia.

Buffers

Buffer A (perfusion buffer) consisted of 140 mM-NaCl, 6 mM-KCl and 10 mM-Hepes adjusted to pH 7.5 with NaOH. Buffer B (incubation buffer) consisted of 120 mM-NaCl, 5 mM-KCl, 1 mM-$MgSO_4$, 25 mM-$NaHCO_3$ and 1 mM-$CaCl_2$, containing 10 mM-glucose. After gassing with O_2/CO_2 (19:1) and addition of highly purified bovine serum albumin (3%, w/v), the buffer was adjusted to pH 7.4. All experiments using intact cells were performed with this buffer.

Preparation of isolated hepatocytes

Isolated hepatocytes from 180–200 g male fed Sprague–Dawley rats (Charles River, France) were prepared by sequential Ca^{2+} chelation and enzymic treatment as described by Seglen (1973), with minor modifications (Postel-Vinay & Desbuquois, 1977). Briefly, the liver was perfused *in situ* through the portal vein at a flow rate of 25 ml/min, first with 150 ml of buffer A containing 0.6 mM-EGTA, and then with 150 ml of buffer A containing 12 mM-$CaCl_2$ and collagenase (0.5 mg/ml). All media were kept at 37 °C. At the end of the perfusion, the liver was carefully removed and gently shaken in 80 ml of buffer B for 2–3 min. The suspension was then filtered through silk (mesh 40 μm), and cells were centrifuged at 1000 *g* for 20 s at room temperature. The supernatant and the upper layer of the cell pellet were removed and cells were washed twice with buffer B. Cells were resuspended in buffer B (5×10^6 cells/ml) and preincubated at 37 °C with gentle shaking under O_2/CO_2 (19:1) for 30 min. The cell suspension was centrifuged at 1000 *g* for 20 s; the supernatant, containing slightly damaged cells, was removed, and the pellet was resuspended in buffer B at a concentration of about 5×10^6 cells/ml. Cells were counted in a haemocytometer. Cell viability, as estimated by the Trypan Blue exclusion method, was 85–95%.

Preparations of ^{125}I-labelled cholera toxin and ^{125}I-labelled protein A

Cholera toxin was labelled with ^{125}I at a specific radioactivity of 800 Ci/mmol as previously described (Janicot & Desbuquois, 1987). Protein A was labelled with ^{125}I by the same procedure, with minor modifications. Protein A (25 μg) was treated, in a 5 ml glass tube, with $Na^{125}I$ (4 MBq; about 0.1 mCi) in the presence of chloramine-T (30 μg) in 0.1 ml of 0.3 M-sodium phosphate buffer, pH 7.4. After 1 min at room temperature, sodium metabisulphite (70 μg in 10 μl of the phosphate buffer) was added, rapidly followed by the addition of 0.2 ml of 50 mM-barbital buffer, pH 8, containing 0.25% (w/v) bovine serum albumin and 0.01% (w/v) NaN_3. ^{125}I-labelled Protein A was purified by chromatography on a 5 ml column of Dowex X8 equilibrated and eluted with the same buffer. It was kept at −20 °C and used within 2 months. The specific radioactivity of the radioiodinated Protein A was about 150 Ci/mmol.

Assay of cell-associated cholera toxin

Total cell-associated (cell-surface-associated plus intracellular) toxin was assayed by using ^{125}I-labelled toxin as a ligand. Hepatocytes (2×10^6 cells/ml) were incubated in polypropylene tubes with 50000 c.p.m. (0.06 pmol) of ^{125}I-labelled cholera toxin in 0.5 ml of buffer B; the time and the temperature of incubation are specified for each particular experiment. The incubation was terminated by adding 1 ml of ice-cold buffer B and centrifuging at 1300 *g* for 1 min. Cells were washed twice with ice-cold buffer B, and the radioactivity bound to cell pellets was determined. Non-specific binding was determined by performing parallel incubations in which excess native toxin (10 μg/ml) was added just before the ^{125}I-toxin.

Assay for cell-surface-associated toxin

This was done radioimmunochemically, with native toxin as a ligand. Hepatocytes (2×10^6 cells/ml) were incubated with native cholera toxin (0.5 μg/ml; about 6 nM) for the indicated times and temperatures, washed twice with ice-cold buffer B to remove free toxin, and resuspended in the original volume of buffer B. Cells were then incubated, in triplicate, with IgG-CT, IgG-CT_A or IgG-CT_B in 0.6 ml of buffer B for 45 min at 4 °C. Cells were then washed twice with ice-cold buffer B to remove free antibody, and, after centrifugation at 1300 *g* for 1 min and resuspension they were incubated with ^{125}I-labelled Protein A (50000 c.p.m.; 250 pmol) in 0.6 ml of buffer B for 45 min at 4 °C. Finally, cells were washed twice with ice-cold buffer B, and cell-associated radioactivity was determined. Non-specific binding was determined by subjecting cells not treated with native cholera toxin to the same procedure, or by omitting the antisera. This non-specific binding represented less than 5% and 0.1% of total radioactivity bound to the hepatocytes in the presence of native toxin and antibody respectively. Control experiments showed that the amount of ^{125}I-labelled Protein A/antibody complexes precipited by poly(ethylene glycol) was the same, regardless of the particular antibody used (results not shown).

Characterization of cell-associated radioactivity

The radioactivity associated with hepatocytes was characterized by one-dimensional discontinuous 10%-polyacrylamide slab- or tube-gel electrophoresis in the presence of SDS as previously described (Laemmli, 1970). Samples were directly dissolved in sample buffer containing SDS and boiled for 2 min before being submitted to electrophoresis.

Assays of cellular cyclic AMP content and adenylate cyclase activity

To assess the effects of cholera toxin on adenylate cyclase, two parameters were measured: on the one hand, cellular cyclic AMP content in whole cells; and on the other hand, the activity of adenylate cyclase in the particulate fraction obtained from these cells. To determine cellular cyclic AMP content, hepatocytes (2×10^6 cells/ml) were preincubated for 20 min at 37 °C in buffer B containing 5 mM-isobutylmethylxanthine (to inhibit cyclic AMP phosphodiesterases). Cholera toxin (10 μg/ml; concentration leading to a maximal response) was then added and the mixture was incubated at 37 °C. At the indicated times, 0.5 ml samples were rapidly removed and centrifuged at 1300 *g* for 1 min; the supernatant was aspirated and cyclic AMP was extracted from the cell pellet by adding 1 ml of ethanol. After incubation for at least 2 h at room temperature, tubes

were centrifuged at 2000 *g* for 1 min, and supernatants containing cyclic AMP were collected and evaporated at room temperature to remove ethanol. The dry residues were then dissolved in 50 mM-Tris/HCl buffer, pH 7.6, containing 5 mM-$MgCl_2$, and assayed for cyclic AMP by a competitive protein-binding assay (Gilman, 1970) using human erythrocyte membranes (Iyengar & Birnbaumer, 1981). Incubation mixtures contained, in 200 μl of the above Tris/$MgCl_2$ buffer, appropriate dilutions of unknown sample or cyclic AMP standards (0.05–10 pmol), 6000 c.p.m. of cyclic [^{3}H]AMP, and enough erythrocyte membranes to bind approx. 60% of the tracer in the absence of added native cyclic AMP. The mixtures were then incubated at 4 °C for 2 h. The reaction was terminated by adding to each tube 1.8 ml of a suspension consisting of 1 ml of poly(ethylene glycol) (25%, w/w) and 0.8 ml of 10 mM-Tris/HCl buffer, pH 7.6, containing 0.33 mg of γ-globulin. After centrifugation for 45 min at 2500 *g*, supernatants were removed and pellets were dissolved at 40 °C in 0.25 ml of 10% SDS. Then 3 ml of Pico-Fluor 30 (Packard) was added, and the membrane-associated radioactivity was determined. To measure adenylate cyclase activity, cells were incubated with native toxin in buffer B by the same procedure as described above, except for the absence of isobutylmethylxanthine. At the indicated times, 1 ml samples of cell suspension were centrifuged at 2000 *g* for 1 min, and resuspended in 5 ml of 50 mM-Tris/HCl buffer, pH 7.6. Cells were then homogenized by using a Polytron homogenizer (10 s at 50% of maximum power). Cell homogenates were centrifuged at 30000 *g* for 30 min, and membrane pellets were washed once in the Tris buffer and stored at −80 °C until assayed for adenylate cyclase activity. The latter was measured by the method of Salomon *et al.* (1974), with minor modifications. The reaction mixture contained, in a final volume of 100 μl: 25 mM-Tris/HCl, pH 7.6, 5 mM-$MgCl_2$, 1 mM-EDTA, 0.2 mg of creatine kinase/ml, 15 mM-phosphocreatine, 0.1 mM-GTP, 1 mM-cyclic [^{3}H]AMP (200 c.p.m./nmol), 0.5 mM-[α-^{32}P]ATP (40–50 c.p.m./pmol) and 1 mg of membrane protein/ml. The reaction was initiated by the addition of membranes, and allowed to proceed at 30 °C for 10 min. The reaction was stopped by the addition of 100 μl of a solution containing 1% SDS, 10 mM-ATP and 2.5 mM-cyclic AMP at pH 7.4, and boiled for 3 min. Assay blanks were prepared by omitting membranes. Cyclic AMP was isolated by the method C described by Salomon *et al.* (1974).

Protein determination

Protein was determined by the Lowry modification of the Folin-Ciocalteu method (Lowry *et al.*, 1951), with crystalline bovine serum albumin as a standard.

RESULTS

Binding of ^{125}I-labelled cholera toxin to isolated hepatocytes

When ^{125}I-toxin (0.12 nM) was incubated with hepatocytes (2×10^6 cells/ml) at 37 °C, the binding of the toxin to cells was half-maximal within 2 min and reached a maximum in 3–5 min (specific and non-specific binding represented about 60% and 10% of the total radioactivity added respectively). This steady state was maintained during a subsequent 90 min incubation period. Decreasing the incubation temperature from 37 °C to 20 °C or 4 °C affected neither the time course of ^{125}I-toxin binding nor the amount of tracer bound to cells at steady state (results not shown). The specific binding of ^{125}I-toxin to isolated hepatocytes was competitively inhibited by native toxin, with half-maximal and maximal effects at approx. 0.1 nM and 10 nM respectively (results not shown). Analysis of these data by Scatchard (1949) plots, illustrated in Fig. 1, revealed a single class of high-affinity binding sites ($K_D \simeq 0.1$ nM; number of sites about 10^7 per cell). Choleragenoid, a biologically inactive protein from *Vibrio cholerae* related to cholera toxin, was equipotent to native toxin in its ability to compete for binding (results not shown).

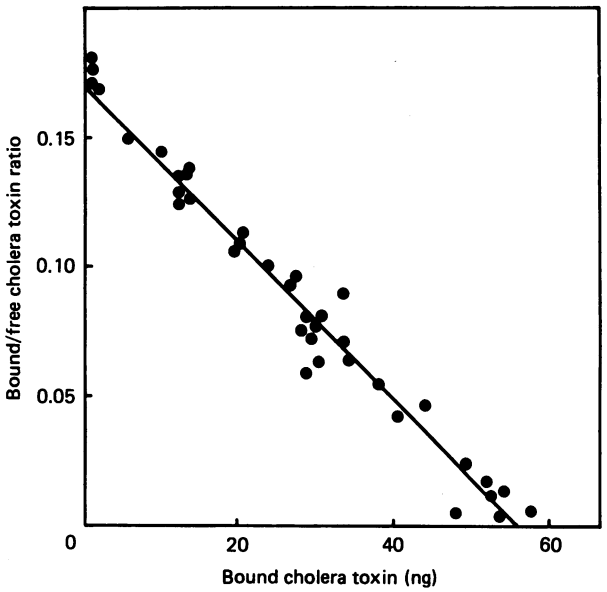

Fig. 1. Scatchard plot of the binding of ^{125}I-labelled cholera toxin to isolated hepatocytes

Isolated hepatocytes (50000 cells), equilibrated at 4 °C, were incubated for 30 min at 4 °C with ^{125}I-labelled cholera toxin (50000 c.p.m.; 0.06 pmol) and increasing amounts of unlabelled toxin (0–10 μg per tube) in 0.6 ml of buffer B. Free and cell-bound toxin were separated as described in the Materials and methods section. The data were analysed and plotted by the method of Scatchard (1949). The results shown in this Figure are means of at least five experiments with separate hepatocyte preparations.

Use of specific antisera to assess the association of cholera toxin with the cell surface

In the experiment described above, it was not possible to discriminate between cell-surface-associated and internalized ^{125}I-toxin. Accordingly, cells were incubated with native cholera toxin, and cell-surface-associated toxin was estimated from its ability to bind to antibodies against the whole toxin or its subunits, by using ^{125}I-labelled Protein A. When isolated hepatocytes were first incubated with native toxin at 4 °C (a temperature at which cholera toxin does not undergo endocytosis), sedimented (to remove unbound toxin), and incubated at 37 °C, a progressive loss of the accessibility of cell-bound toxin to antibody occurred with time (Fig. 2). The rate and extent of this process were highest with IgG-CT_A, intermediate with IgG-CT and lowest with IgG-CT_B, with about 25, 50 and 70% of initially bound toxin

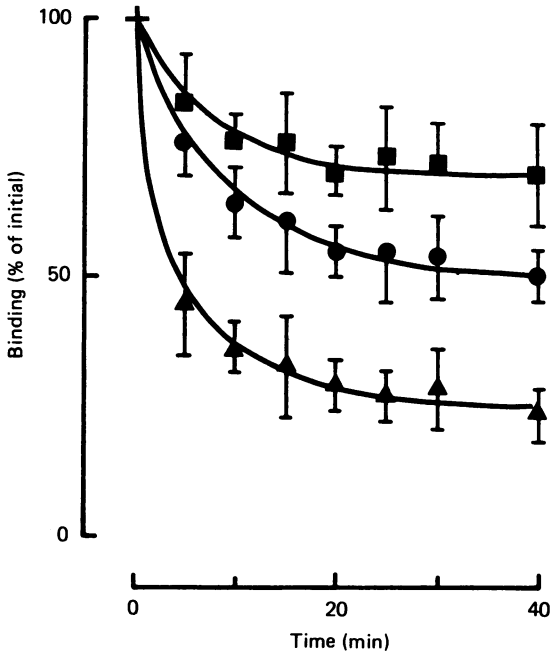

Fig. 2. Changes in surface-associated cholera toxin in hepatocytes as a function of time at 37 °C

Isolated hepatocytes (2×10^6 cells/ml) were first equilibrated at 4 °C and preincubated with 0.5 μg of native cholera toxin/ml in buffer B for 15 min at 4 °C. Then 0.5 ml samples of this suspension were centrifuged; cells were resuspended in the original volume of buffer B and transferred at 37 °C. At the indicated times, cell-surface-associated toxin was measured by using specific antibodies against whole toxin (●), A subunit (▲) or B subunit (■), as described in the Materials and methods section. Results are expressed as the percentage of cell-associated toxin bound to antibody at zero time, and are means ± S.E.M. for four to six separate experiments.

remaining accessible to antibody after 40 min respectively. As bound toxin did not dissociate from the cells during incubation at 37 °C (results not shown), these results can be attributed either to penetration of the toxin into the plasma membrane or to its endocytosis into the cells.

Effect of pH of the medium on the association of cholera toxin with the cell surface

Previous studies with diphteria toxin have shown that, although entry of this toxin into cells normally occurs by adsorptive endocytosis, exposure of cells to low pH allows direct penetration of the toxin through the plasma membrane (Draper & Simon, 1980; Sandvig & Olsnes, 1980). To determine whether a similar phenomenon occurred with cholera toxin, the effects of pH on the accessibility of cell-associated toxin to anti-toxin antibodies were examined. These experiments were performed at 4 °C (to prevent endocytosis of the toxin). In a first series of experiments, cells were incubated with native cholera toxin in medium of neutral pH, washed to remove unbound toxin, and incubated for various times in media of neutral (pH 7.4) or acid (pH 5) pH (Fig. 3). Whereas the accessibility of cell-associated toxin to antibody was unchanged under neutral incubation conditions, it progressively decreased with time under acidic conditions. The rate and extent of this process were higher with IgG-CT_A than with IgG-CT, with about 50% and 80% of toxin initially bound remaining accessible to antibody at 40 min respectively. With IgG-CT_B, only a slight and late decrease in accessibility was observed. In a second series of experiments, cells preincubated with native cholera toxin and washed as described above were incubated for 60 min in media of various pH values (Fig. 4). Accessibility of cell-associated toxin to antibody was essentially 100% at pH 7.4 and 6.0, but gradually decreased when the pH was lowered from 6 to 4, with a minimum in the pH range 4–5. Again, this change was higher with IgG-CT_A than with IgG-CT and IgG-CT_B, with about 45, 80 and 90% of initially bound toxin remaining accessible to antibody respectively. Control experiments showed that lowering the pH did not cause dissociation of bound toxin from cells. The data therefore indicate that exposure of cells to an acidic environment facilitates the insertion of toxin into, or its translocation across, the plasma membrane.

Time- and temperature-dependence of the generation of the A_1 peptide and the activation of adenylate cyclase by cholera toxin at neutral and acid pH

It is well established that, in cholera-toxin-treated cells, both the generation of A_1 peptide from the A subunit and the activation of adenylate cyclase exhibit a lag phase, and it has been postulated that, during this period, cholera toxin (or some part of it) undergoes translocation through the plasma membrane (Kassis *et al.*, 1982). Accordingly, the time- and temperature-dependence of these processes in cholera-toxin-treated hepatocytes were examined, under both neutral and acidic incubation conditions. In agreement with a previous report (Houslay & Elliott, 1979), stimulation of adenylate cyclase by cholera toxin at 37 °C occurred after a lag phase of 10 min and reached a maximum (8-fold basal activity) by 40–60 min (Fig. 5*a*). At 20 °C the lag phase of activation was lengthened (30 min) and the increase in activity was decreased (2.5-fold), and at 4 °C no activation was observed. Generation of the A_1 peptide from ^{125}I-labelled cholera toxin at 37 °C and 20 °C occurred with a comparable lag phase (Fig. 5*b*), but, unlike activation of adenylate cyclase, proceeded linearly with time, with about 2 and 1% of bound cholera toxin generated per 30 min respectively. When adenylate cyclase activity was plotted against the percentage of cell-associated toxin recovered as A_1 peptide (Fig. 6), a linear relationship between these two parameters was observed up to 2–3% of A_1 peptide generated (about 2×10^5–3×10^5 molecules per cell). Neither the time course nor the extent of adenylate cyclase activation and of generation of the A_1 peptide at 37 °C were affected by prior exposure of hepatocytes to a medium of pH 5 at 4 °C, or by direct incubation in this medium at 37 °C (results not shown). Thus, although facilitating the insertion of cholera toxin into (or its translocation across) the plasma membrane, exposure of cells to an acidic environment does not increase the generation of the A_1 peptide and the ensuing activation of adenylate cyclase.

Effects of chloroquine and monensin treatments on the generation of A_1 peptide and the activation of adenylate cyclase by cholera toxin

We have previously shown that, in intact liver, generation of the A_1 peptide from cholera toxin occurs

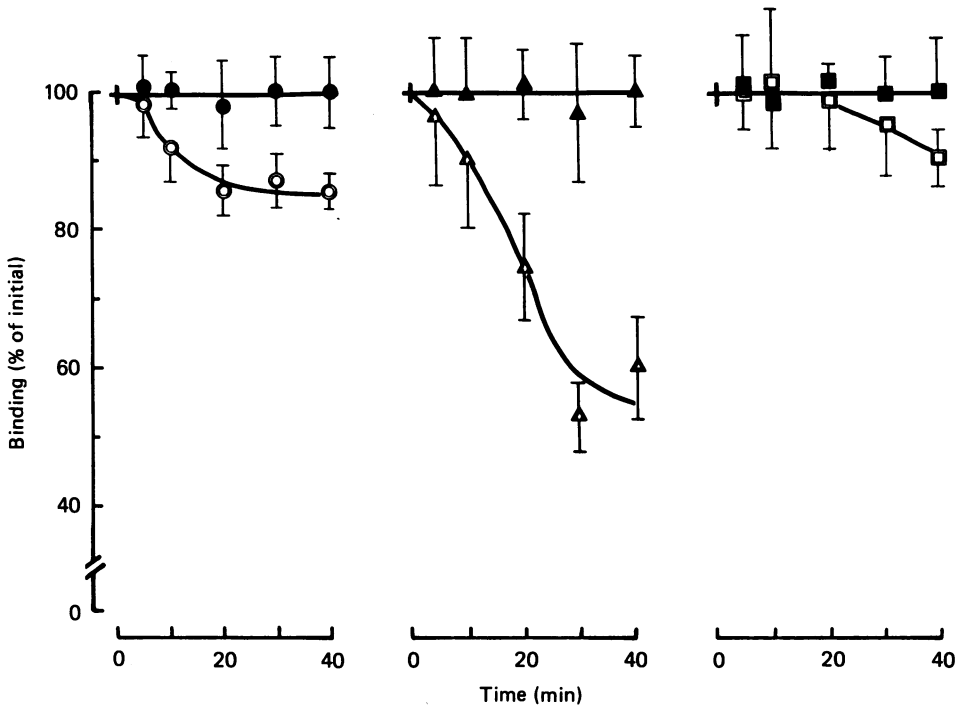

Fig. 3. Changes in surface-associated cholera toxin in hepatocytes exposed to neutral and acidic media as a function of time at 4 °C

Isolated hepatocytes were first equilibrated at 4 °C and preincubated with 0.5 μg of native cholera toxin/ml in buffer B for 15 min at same temperature. Then 0.5 ml samples of this suspension (2×10^6 cells/ml of buffer B) were centrifuged. Cells were resuspended at the same concentration in buffer B (●, ▲, ■) or in buffer B adjusted to pH 5 with HCl (○, △, □) and incubated further at 4 °C. At the indicated times, cell-surface toxin was measured by using specific antibodies against whole toxin (●, ○), A subunit (▲, △) or B subunit (■, □), as described in the Materials and methods section. Results are expressed as indicated in the legend to Fig. 2 and are means ± S.E.M. for three separate experiments.

mainly, if not exclusively, in the endosomal compartment (Janicot & Desbuquois, 1987). To determine whether the low pH of the endosomal content is required for toxin processing and action, the effects of chloroquine, an acidotropic agent, and monensin, a proton ionophore (Mellman *et al.*, 1986), on the generation of the A_1 peptide and the activation of adenylate cyclase were studied (Fig. 7). In these experiments, cellular cyclic AMP content was also measured. In control hepatocytes, cellular cyclic AMP content increased 20 min after addition of cholera toxin and reached a maximum (125-fold over basal) at 40–60 min (Fig. 7*b*); the time-dependences of the generation of the A_1 peptide and of the stimulation of adenylate cyclase activity (Figs. 7*a* and 7*c*) were as described above. Chloroquine and monensin treatments both lengthened the lag phase and decreased the rate at which cholera toxin stimulated adenylate cyclase activity and increased cellular cyclic AMP content. These drugs were equally effective in inhibiting adenylate cyclase activity (about 50% inhibition), but chloroquine was more effective than monensin in inhibiting cyclic AMP production (about 60 and 40% inhibition respectively). Chloroquine and monensin treatments also decreased the rate of generation of the A_1 peptide, but in this case, monensin was more effective than chloroquine (about 75 and 50% inhibition respectively). As with control hepatocytes, adenylate cyclase activity in chloroquine- and monensin-treated hepatocytes was directly correlated with the percentage of A_1 peptide generated (Fig. 8). However, for a given amount of A_1 peptide generated, adenylate cyclase activity was increased in monensin-treated cells, and, conversely, decreased in chloroquine-treated cells. In isolated plasma membranes, monensin also increased, and chloroquine also decreased, the stimulation of adenylate cyclase activity by dithiothreitol-reduced cholera toxin (results not shown). Thus, in addition to their ability to inhibit the generation of A_1 peptide in intact cells, chloroquine and monensin appear to affect membrane-associated step(s) involved in the activation of adenylate cyclase by cholera toxin, albeit in opposite ways. Exposure of chloroquine-treated cells to acidic conditions (pH 5) affected neither the generation of A_1 peptide nor the activation of adenylate cyclase by cholera toxin (results not shown).

Effects of chloroquine and monensin treatments on the association of cholera toxin with the cell surface

Although consistent with the view that chloroquine and monensin treatments inhibit the generation of the A_1 peptide by increasing the endosomal pH, the results described above do not exclude the possibility that these drugs inhibit the delivery of the toxin to the endosomal compartment. Accordingly, the effects of chloroquine and monensin on the endocytosis of cell-bound toxin were examined by using IgG-CT_B. As shown in Fig. 9, although the accessibility of bound toxin to IgG-CT_B in chloroquine- and monensin-treated cells decreased with time, the rate and extent of this process were markedly lower than in control hepatocytes. Assuming that the

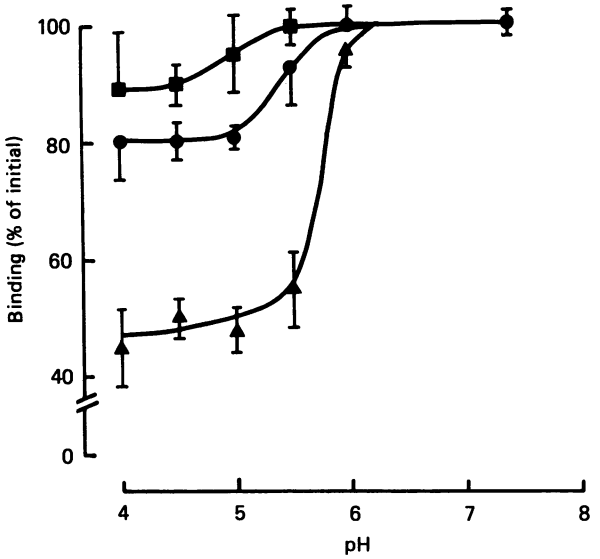

Fig. 4. Changes in surface-associated cholera toxin in hepatocytes as a function of pH of the medium at 4 °C

Isolated hepatocytes were first equilibrated at 4 °C and preincubated with 0.5 μg of native cholera toxin/ml at 4 °C for 15 min. Then 0.5 ml samples of hepatocytes (2×10^6 cells/ml of buffer B) were centrifuged. Cells were resuspended at the same concentration in buffer B adjusted to the indicated pH with HCl, and incubated at 4 °C for 60 min. Cell-surface toxin was measured by using specific antibodies against the whole toxin (●), A subunit (▲) or B subunit (■), as described in the Materials and methods section. Results are expressed as indicated in the legend to Fig. 2 and are means ± S.E.M. for three separate experiments.

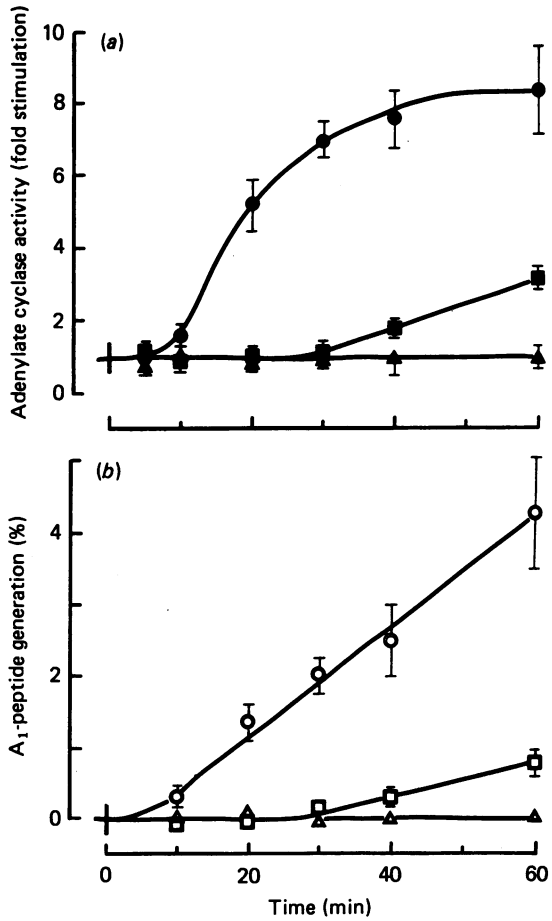

Fig. 5. Time course of activation of adenylate cyclase (*a*) and of generation of A_1 peptide (*b*) in cholera-toxin-treated hepatocytes

(*a*) Isolated hepatocytes (2×10^6 cells/ml) were first equilibrated at 37 °C (●), 20 °C (■) and 4 °C (▲), and incubated with 10 μg of cholera toxin/ml at the same temperatures. At the indicated times, 1 ml samples (in triplicate) of these cell suspensions were centrifuged. Cells were homogenized and adenylate cyclase activity in the cell particulate fraction was assayed as described in the Materials and methods section. Results are expressed by reference to basal (unstimulated) activity (1 pmol/min per mg of protein) and are means ± S.E.M. for four to five separate experiments. (*b*) Isolated hepatocytes (10^6 cells/ml) were first equilibrated at 4 °C and incubated with ^{125}I-labelled cholera toxin (10^6 c.p.m., representing about 1 pmol) for 15 min at the same temperature. After centrifugation to remove free ^{125}I-toxin, cells were resuspended in the original volume and transferred at 37 °C (○), 20 °C (□) and 4 °C (△). At the indicated times, 1 ml samples (triplicate) of cell suspensions were centrifuged; pellets were dissolved in electrophoresis sample buffer, and the radioactivity was characterized as described in the Materials and methods section. Results are expressed as the percentage of cell-associated toxin, and have been corrected for the small amount of A_1 peptide (0.3%) present at zero time (before transfer at different temperatures). They are means ± S.E.M. for five to six separate experiments.

change in accessibility of bound toxin to IgG-CT_B reflects primarily a change in endocytosis, it can be estimated that monensin and chloroquine inhibit the rate of endocytosis of the toxin by 4- and 2-fold respectively. Furthermore, at each time point examined, the endocytosis of cell-bound toxin was inhibited exactly to the same extent as generation of A_1 peptide. Thus it appears that the ability of chloroquine and monensin treatments to inhibit the generation of the A_1 peptide results predominantly, if not exclusively, from an inhibition of the endocytosis of the toxin.

DISCUSSION

The initial step in the interaction of cholera toxin with cells is its binding to specific cell-surface receptors (Bennett & Cuatrecasas, 1976). These studies show that the binding of cholera toxin to isolated rat hepatocytes is rapid, specific and saturable, and involves a single class of homogeneous binding sites. At 4 °C, a temperature at which cell-associated toxin does not undergo internalization, about 10^7 sites per cell with a dissociation constant (K_D) of 0.1 nM have been identified. The binding capacity of hepatocytes was one to three orders of magnitude higher, and the dissociation constant of the hepatocyte-toxin interaction was one to two orders of magnitude lower, than found with other cell types (for discussion, see Hyun & Kimmich, 1984). The former observation may reflect a higher content of hepatocytes in ganglioside GM_1. In addition, when related to protein, the binding capacity of hepatocytes (about 40 pmol/mg) was higher than that reported (10 pmol/mg) for an equivalent amount of liver microsomal membranes (Cuatrecasas, 1973*a*). This may reflect in part the greater

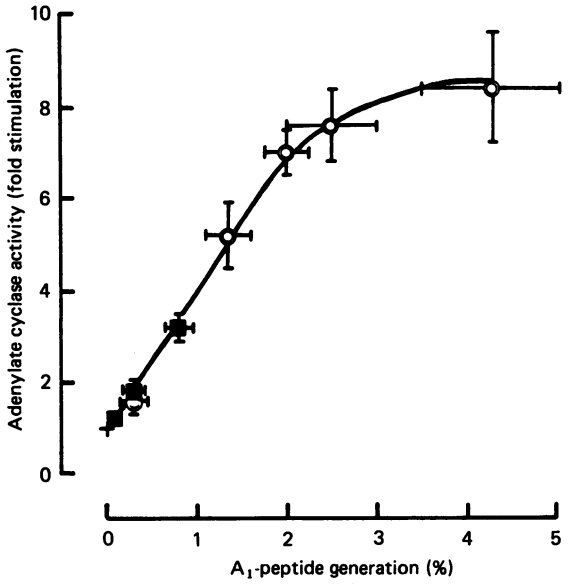

Fig. 6. Activation of adenylate cyclase as a function of A_1-peptide generation in cholera-toxin-treated hepatocytes

This Figure was constructed from the data shown in Fig. 5 obtained at 37 °C (○) and 20 °C (■). Results for A_1-peptide generation and for adenylate cyclase activity are expressed as means ± S.E.M.

binding capacity of hepatocytes relative to other types of liver cells, and the incomplete recovery of surface binding sites in the microsomal fraction.

In several cell types examined, cell-associated cholera toxin has been shown to undergo a time- and temperature-dependent internalization, and morphological evidence that this process involves a redistribution of the toxin within the plasma membrane (Craig & Cuatrecasas, 1975; Hansson *et al.*, 1977) or its endocytosis (Joseph *et al.*, 1978, 1979; Montesano *et al.*, 1982) has been presented. In agreement with Fishman's (1982) studies on neuroblastoma cells, we have found that, at 37 °C, the accessibility of cell-associated toxin to anti-toxin antibody progressively decreases with time. Furthermore, toxin accessibility to antibodies against isolated toxin subunits also decreased, this change occurring more rapidly and to a greater extent with antibody against the A subunit than with antibody against the B subunit. Since A and B subunits undergo stoichiometric endocytosis in liver *in vivo*, these results suggest that toxin internalization into isolated hepatocytes may involve two distinct pathways: on the one hand, an endocytosis of the whole toxin, reflected by a parallel decrease in the accessibility of A and B subunits to their respective antibodies; and on the other hand, a further process that causes a selective decrease in the accessibility of the A subunit. That this second pathway involves a penetration of the A subunit into the plasma membrane is suggested by the photolabelling studies by Wisnieski & Bramhall (1981) and Tomazi & Montecucco (1981), carried out on Newcastle-disease virus and GM_1-containing liposomes respectively.

One major step in cholera-toxin action on cells is the generation of the A_1 peptide from the A subunit (Kassis *et al.*, 1982). We have found that this process is time- and temperature-dependent, occurring after a lag phase and proceeding linearly with time. Importantly, generation of

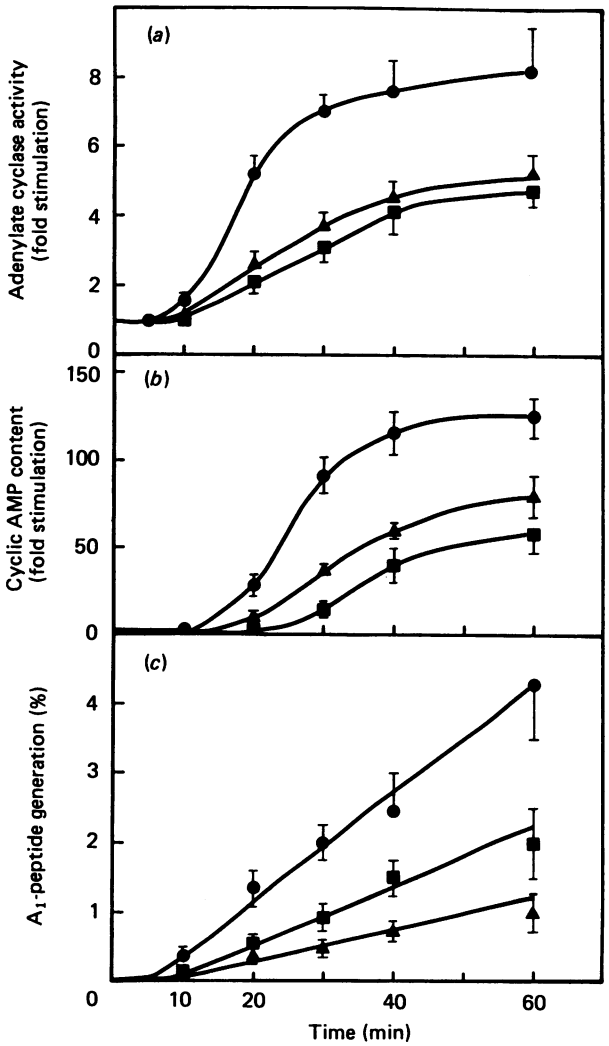

Fig. 7. Effect of chloroquine and monensin on the time course of adenylate cyclase activation (*a*), cellular cyclic AMP content increase (*b*) and A_1-peptide generation (*c*) in cholera-toxin-treated hepatocytes

Isolated hepatocytes were incubated at 37 °C in the absence (●) or in the presence of 0.4 mM-chloroquine (■) or 10 μM-monensin (▲) for the indicated times. Adenylate cyclase activity and A_1-peptide generation were measured and expressed as described in the Materials and methods section and in the legend to Fig. 5; cellular cyclic AMP content was measured as described in the Materials and methods section and expressed by reference to basal content (about 1 pmol/mg of protein or 10^6 cells). The results for each parameter are expressed as means ± S.E.M. for three to five separate experiments.

the A_1 peptide did not occur until a substantial fraction of cell-associated toxin had disappeared from the cell surface, in agreement with our previous finding that only in the endosomal compartment is the A_1 peptide generated *in vivo*. In addition, at any given time, the fraction of cell-associated toxin that was converted into A_1 peptide was much lower than that which had lost accessibility to antibody against the A subunit. This suggests that, unlike the observations by Tomazi & Montecucco (1981) with GM_1-containing liposomes, reduction is not required for the penetration of the A subunit into the plasma membrane of liver cells.

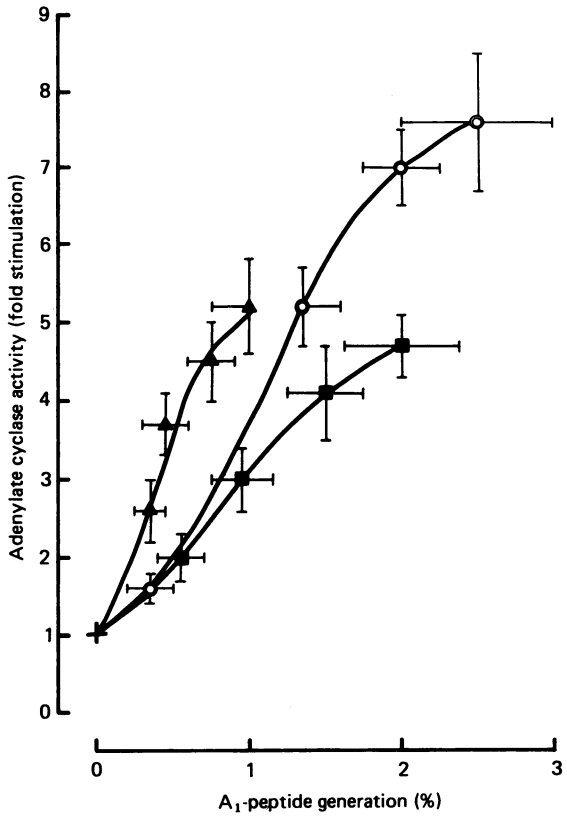

Fig. 8. Adenylate cyclase activation as a function of A_1-peptide generation in cholera-toxin-treated hepatocytes, in the absence or in the presence of chloroquine or monensin

This Figure was contructed from the data shown in Figs. 7(*a*) and 7(*c*) in the absence (○) or in presence of chloroquine (■) or monensin (▲). Results are expressed as indicated in the legend to Fig. 7.

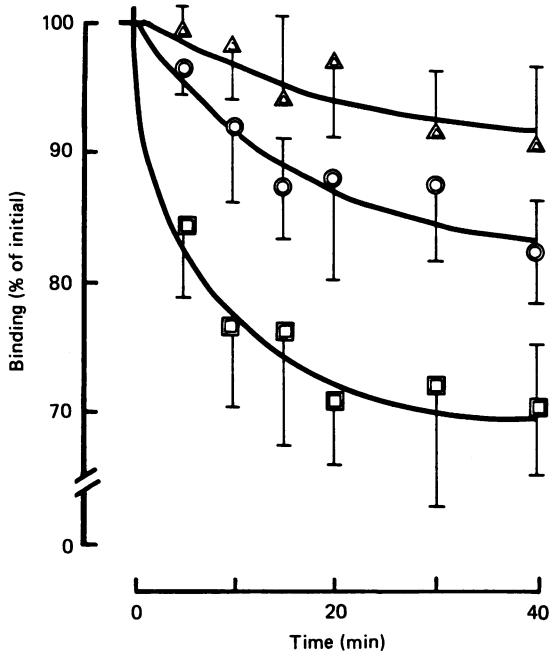

Fig. 9. Effect of chloroquine and monensin on the time course of disappearance of cholera toxin from the hepatocyte surface

After equilibration in the absence or in the presence of drugs at 4 °C, hepatocytes were first incubated with 0.5 μg of native cholera toxin/ml in the absence (□) or in the presence of 10 μM-monensin (△) or 0.4 mM-chloroquine (○) in buffer B for 15 min. Hepatocytes were then centrifuged, resuspended in buffer B in the absence or in the presence of the drugs and transferred at 37 °C. At the indicated times, cell-surface-associated cholera toxin was measured by using specific antibody against the B subunit as described in the Materials and methods section. Results are expressed as indicated in the legend to Fig. 3.

The role of the A_1 peptide as the activator of adenylate cyclase in cholera-toxin-treated cells is well established. As in other cell types (Kassis *et al.*, 1982), activation of adenylate cyclase in hepatocytes kinetically paralleled the generation of the A_1 peptide and showed the same temperature-dependence, confirming that these processes are functionally linked. However, although adenylate cyclase activity was linearly related to the fractional generation of the A_1 peptide up to 2–3% of cell-associated toxin, no further increase in enzyme activity occurred above this value. This finding is consistent with previous reports showing that concentrations of cholera toxin required for maximal activation of adenylate cyclase or cyclic AMP production in cells are several orders of magnitude lower than concentrations required for full occupancy of toxin-binding sites, and that a minor fraction of cell-associated toxin is involved in enzyme activation (Bennett & Cuatrecasas, 1976).

A number of animal viruses, as well as many bacterial and plant toxins, are known to enter cells by adsorptive endocytosis (Marsh, 1984; Mellman *et al.*, 1986). However, at least with Semliki Forest virus (Helenius *et al.*, 1980), polio virus type I (Madshus *et al.*, 1984) and diphtheria toxin (Draper & Simon, 1980; Sandvig & Olsnes, 1980), lowering the pH of the medium has been shown to allow direct penetration of these ligands through the plasma membrane, presumably by mimicking the acidic environment achieved in the endosomal compartment. In the present studies, exposure of hepatocytes to a low pH caused a marked and selective decrease in the accessibility of cell-associated toxin to antibody against the A subunit. Since this change occurred at 4 °C, a temperature at which toxin does not undergo endocytosis, the mechanism involved is likely to be an enhanced penetration of the A subunit into the plasma membrane. However, exposure of hepatocytes to an acidic medium failed to increase the generation of the A_1 peptide, and, unlike what was observed with diphtheria toxin, to augment toxin cytotoxicity. These observations are consistent with the failure of isolated liver plasma-membrane fractions to catalyse the reduction of the A subunit of the toxin (M. Janicot, unpublished work).

The concept that many viruses and protein toxins enter the cell cytoplasm by adsorptive endocytosis is largely based on the ability of acidotropic drugs and carboxylic ionophores to protect cells from viral infection or toxin cytotoxicity respectively (Mellman *et al.*, 1986). These drugs are believed to inhibit ligand fusion with and translocation across the endosomal membrane, by virtue of their ability to elevate the endosomal pH. In the present study, we have confirmed that chloroquine inhibits the ability of cholera toxin to activate adenylate cyclase (Houslay & Elliott, 1981) and have found that a

similar effect occurs with monensin. In addition, both drugs inhibited to approximately the same extent the generation of the A_1 peptide. However, a somewhat unexpected effect of chloroquine and monensin was also to inhibit the endocytosis of cell-associated toxin, as judged by accessibility to antibody against the B subunit. This observation suggests that the decreased generation of the A_1 peptide in chloroquine- and monensin-treated hepatocytes does not result from an increase in endosomal pH, but rather from a decrease in toxin endocytosis. Although the main effects of chloroquine and monensin are to inhibit the dissociation of internalized ligand–receptor complexes and the recycling of internalized receptors, occasional effects of these drugs on fluid-phase (Wilcox *et al.*, 1982) and receptor-mediated endocytosis (Sandvig *et al.*, 1979; Schlegel *et al.*, 1981; Marsh *et al.*, 1982; Keusch & Jacewicz, 1984; Sorimachi *et al.*, 1985) have also been described.

In summary, these studies show that the endocytosis of cholera toxin is required for generation of the A_1 peptide and subsequent activation of adenylate cyclase in isolated rat hepatocytes. They also show that a low pH facilitates the penetration of the A subunit into the plasma membrane, suggesting that acidity may be required for translocation of the A subunit (or A_1 peptide) across the endosomal membrane as well.

We thank F. Gehin-Fouque for her expert technical assistance and Dr. P. Dodin for his advice and for his generous gift of antitoxin antisera.

REFERENCES

Bennett, V. & Cuatrecasas, P. (1976) in The Specificity and Action of Animal, Bacterial and Plant Toxins: Receptors and Recognition, Series B, Volume 1 (Cuatrecasas, P., ed.), pp. 131–156, Chapman and Hall, London
Cassel, D. & Pfeuffer, T. (1978) Proc. Natl. Acad. Sci. U.S.A. **75**, 2669–2673
Craig, S. W. & Cuatrecasas, P. (1975) Proc. Natl. Acad. Sci. U.S.A. **72**, 3844–3848
Cuatrecasas, P. (1973*a*) Biochemistry **12**, 3547–3558
Cuatrecasas, P. (1973*b*) Biochemistry **12**, 3558–3566
Draper, R. K. & Simon, M. I. (1980) J. Cell Biol. **87**, 849–854
Finkelstein, R. A. (1973) CRC Crit. Rev. Microbiol. **2**, 553–623
Fishman, P. H. (1982) J. Cell Biol. **93**, 860–865
Gill, D. M. & Meren, R. (1978) Proc. Natl. Acad. Sci. U.S.A. **75**, 3050–3054
Gilman, A. G. (1970) Proc. Natl. Acad. Sci. U.S.A. **67**, 305–312
Hansson, H. A., Holgren, J. & Svennerholm, L. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 3782–3786
Helenius, A., Kartenbeck, J., Simons, K. & Fries, E. (1980) J. Cell Biol. **84**, 404–420
Holgrem, J. (1981) Nature (London) **292**, 413–417
Houslay, M. D. & Elliott, K. R. F. (1979) FEBS Lett. **104**, 359–363
Houslay, M. D. & Elliott, K. R. F. (1981) FEBS Lett. **128**, 289–292
Hyun, C. S. & Kimmich, G. A. (1984) Am. J. Physiol. G623–G631
Iyengar, R. & Birnbaumer, L. (1981) in Laboratory Methods Manual for Hormone Action and Molecular Endocrinology (Schrader, W. T. & O'Malley, B. W., eds.), pp. 9–37, Biological Associates, Houston
Janicot, M. & Desbuquois, B. (1987) Eur. J. Biochem. **163**, 433–442
Joseph, K. C., Seung, U. K., Stieber, A. & Gonatas, N. K. (1978) Proc. Natl. Acad. Sci. U.S.A. **75**, 2815–2819
Joseph, K. C., Stieber, A. & Gonatas, N. K. (1979) J. Cell Biol. **81**, 543–554
Kassis, S., Hagmann, J., Fishman, P. H., Chang, P. P. & Moss, J. (1982) J. Biol. Chem. **257**, 12148–12152
Keusch, G. T. & Jacewicz, M. (1984) Biochem. Biophys. Res. Commun. **121**, 69–76
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
Madshus, I. H., Olsnes, S. & Sandvig, K. (1984) J. Cell Biol. **98**, 1194–1200
Marsh, M. (1984) Biochem. J. **218**, 1–10
Marsh, M., Wellsteed, J., Kern, H., Harms, E. & Helenius, A. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 5297–5301
Mellman, I., Fuchs, R. & Helenius, A. (1986) Annu. Rev. Biochem. **55**, 663–700
Montesano, R., Roth, J., Robert, A. & Orci, L. (1982) Nature (London) **296**, 651–653
Moss, J. & Vaughan, M. (1979) Annu. Rev. Biochem. **48**, 581–600
Moss, J., Stanley, S. J. & Lin, M. C. (1979) J. Biol. Chem. **254**, 11993–11996
Postel-Vinay, M. C. & Desbuquois, B. (1977) Endocrinology (Baltimore) **100**, 209–215
Salomon, Y., Londos, C. & Rodbell, M. (1974) Anal. Biochem. **58**, 541–548
Sandvig, K. & Olsnes, S. (1980) J. Cell Biol. **87**, 828–832
Sandvig, K., Olsnes, S. & Pihl, A. (1979) Biochem. Biophys. Res. Commun. **90**, 648–655
Scatchard, G. (1949) Ann. N.Y. Acad. Sci. **51**, 660–672
Schlegel, R., Willingham, M. & Pastan, I. (1981) Biochem. Biophys. Res. Commun. **102**, 992–998
Seglen, P. O. (1973) Exp. Cell Res. **82**, 391–398
Sorimachi, K., Yasumura, Y. & Okayasu, T. (1985) Jpn. J. Exp. Med. **55**, 53–59
Tomazi, M. & Montecucco, C. (1981) J. Biol. Chem. **256**, 11177–11181
van Heyningen, S. (1976) J. Infect. Dis. **133**, S5–S13
Wilcox, D. K., Kitson, R. P. & Widnell, C. C. (1982) J. Cell Biol. **92**, 859–864
Wisnieski, B. J. & Bramhall, J. S. (1981) Nature (London) **289**, 319–321

Received 5 January 1988/11 March 1988; accepted 24 March 1988