Action of activated 27 000 M_r toxin from *Bacillus thuringiensis* var. *israelensis* on Malpighian tubules of the insect, *Rhodnius prolixus*

S. H. P. MADDRELL, J. A. OVERTON

AFRC Unit of Insect Neurophysiology and Pharmacology, Department of Zoology, Downing Street, Cambridge CB2 3EJ, England

D. J. ELLAR and B. H. KNOWLES

Department of Biochemistry, Tennis Court Road, Cambridge CB2 1QW, England

Summary

The action of activated 27 000 M_r toxin from *Bacillus thuringiensis* var. *israelensis* (Bti toxin) on Malpighian tubules of *Rhodnius prolixus* has been investigated. Its binding to the tubules is slowed by low temperature but is not prevented even at 0°C. The binding is less effective at pH 10 than at pH 7. Pretreatment of the tubules with 0.1 mmol l^{-1} ouabain or bumetanide or 1 μmol l^{-1} 5-hydroxytryptamine did not affect the toxicity of the toxin. The toxin causes very large changes in the trans-epithelial potential difference; it changes from 40 mV, lumen negative, often to more than 100 mV, lumen positive. This reflects an initial collapse of the potential of the basal cell membrane, followed by a large positive-going potential change at the luminal cell membrane. Just prior to the effects of the toxin on rapid fluid secretion, the basal cell membrane becomes permeable to sucrose molecules. Raffinose at 170 mmol l^{-1} in the bathing solution does not protect the tubules from Bti toxin action but dextran, M_r 5000, at 60 mmol l^{-1} significantly delayed failure of fluid secretion and, even more, the onset of staining of the tubule cells with Trypan Blue. Exposing tubules to saline that is calcium-free and/or magnesium-free, or has a composition adjusted to be similar to that of the intracellular milieu, does not affect the time course of failure of fluid secretion induced by the toxin. There is no evidence that effective aggregates of Bti toxin molecules are formed in concentrated solutions.

The time courses of failure of fluid secretion induced by Bti toxin and melittin differ. Melittin action also involves a delay before effects are observable, but its effects then develop more slowly than with Bti toxin. This is consistent with the suggestion that melittin and Bti toxin act by forming similarly sized pores but that tetramers of melittin form pores while a larger number of molecules of Bti toxin are required to form a pore.

Key words: *Bacillus thuringiensis*, Malpighian tubules, toxin, toxin action, *Rhodnius prolixus*.

Introduction

The 27 000 M_r toxin from *Bacillus thuringiensis* var. *israelensis* (Bti toxin) is normally only toxic to dipteran insects; however, when activated, it has cytolytic effects on many different cells from a wide variety of organisms (Thomas and Ellar, 1983). We have recently studied the kinetics of binding of this activated toxin to cell membranes using, as a model system, the Malpighian tubules of the insect, *Rhodnius prolixus* (Maddrell *et al.* 1988). The results suggest that several toxin molecules need to bind close to each other on the membrane as a complex or aggregate before any toxic effects develop. We now describe factors that affect the rate and extent of this initial binding and provide evidence to support the idea that toxic effects develop through the formation of small pores in the basal cell membranes. There is increasingly good evidence that melittin, the major toxin of bee venom, produces its effects by forming pores in the membranes of cells exposed to it (Vogel and Jahnig, 1986). We report here experiments to compare the effects of melittin with Bti toxin. Finally, we have investigated the possibility that high concentrations of Bti toxin in solution might lead to the formation there of aggregates that could then rapidly insert into the membranes of cells put into the solution, without the need for the complex or aggregate to assemble on the cell surface.

Materials and methods

The insects used were unfed 5th stage larvae of the blood-

Journal of Cell Science 94, 601–608 (1989)

sucking insect, *Rhodnius prolixus* (Hemiptera) from a laboratory culture maintained at 27°C.

Most experiments involved determinations of rates of fluid secretion by Malpighian tubules isolated from these insects. The technique involves observation of the tubules in drops of saline under liquid paraffin (mineral oil). The procedure has been described in detail by Maddrell (1980) and in its recently improved form by Maddrell *et al.* (1988). The standard saline used had the following composition: NaCl, 142 mmol l^{-1}; KCl, 8.6 mmol l^{-1}; $CaCl_2$, 2 mmol l^{-1}; $MgCl_2$, 8.5 mmol l^{-1}; glucose, 34 mmol l^{-1}; Hepes, 8.6 mmol l^{-1}; pH adjusted to 7 with M-NaOH (about 3 cm^3 l^{-1}). To stimulate rapid fluid secretion by the tubules, 5-hydroxytryptamine (5-HT) was included in the saline at 10^{-6} mol l^{-1} (Maddrell *et al.* 1971). We also used a saline (Burgess *et al.* 1983) whose composition was adjusted to that typical of the intracellular milieu. It contained 20 mmol l^{-1} Na, 100 mmol l^{-1} K and 5 mmol l^{-1} Mg. In addition, this cytosolic solution had its free calcium concentration buffered at around 100 nmol l^{-1} and it contained 1.5 mmol l^{-1} ATP maintained with 5 mmol l^{-1} creatine phosphate and 5 units cm^{-3} of creatine kinase; it also contained 2% bovine serum albumin.

To record the trans-epithelial potential of an isolated tubule, glass pipettes pulled out to a tip diameter of 100–200 μm and filled with 3 mol l^{-1} KCl–agar were put, one in the drop bathing the tubule and one in the drop of secreted fluid. Each pipette was connected through plastic tubes filled with KCl–agar to half calomel cells. The potential difference between the calomel cells was measured with a Keithley Electrometer model 602 and recorded on a Servoscribe twin-channel chart recorder. Basal cell membrane potentials were measured using intracellular microelectrodes as described by O'Donnell and Maddrell (1984).

Samples of Bti toxin were prepared as follows. Cloned Bti 27 000 M_r toxin was purified from recombinant *Bacillus subtilis* (Ward *et al.* 1986). Samples (100 μg) of the pure toxin were suspended in 100 or 200 μl of 50 mmol l^{-1} Na_2CO_3, whose pH had been adjusted to 10.5. After brief sonication, the toxin-containing solution was incubated at 37°C for 1 h. A 45 μl sample of the solution was then thoroughly mixed with 5 μl of an extract of gut enzymes from larvae of *Pieris brassicae* (Knowles *et al.* 1984) and incubated at 37°C for 15 min. This ensures the activation of the toxin, yielding a 25 000 M_r polypeptide, which is the ultimate active agent of the toxin (Davidson and Yamamoto, 1984). This stock solution was kept on ice and samples were taken and diluted for use with appropriate amounts of standard saline containing 5-HT. Different final concentrations of toxin were used to produce quick effects or a more delayed onset of failure (Maddrell *et al.* 1988).

To determine the effects of Bti toxin on Malpighian tubules we routinely either followed the rate of fluid secretion or measured the trans-epithelial electrical potential difference (TEP). For some experiments this was not appropriate. Instead we used Trypan Blue staining of the cells. The dye, Trypan Blue, M_r 961, cannot normally enter live cells. However, if the cell membrane is sufficiently damaged, the dye enters and stains the cell contents. The dye is much used in toxin studies to indicate cell death (Knowles and Ellar, 1987). For our experiments, we used Trypan Blue at a concentration of 0.35 mmol l^{-1} (0.03%, w/v).

Tubules were exposed to low temperatures between 0°C and 8°C in culture cabinets belonging to the CRC DNA Repair Unit at the Department of Zoology, Cambridge.

To measure the uptake of sucrose, M_r 342, into tubule cells during toxin action, we immersed tubules in saline containing Bti toxin and [^{14}C]sucrose at 20 000 cts min^{-1} μl^{-1} for different times. The rates of fluid secretion were followed until the tubules were removed into a saline wash for 30 s before each was placed in a 20 μl drop of distilled water. After 30 min, each drop and tubule was transferred to a vial of scintillation fluid for determination of the extent of [^{14}C]sucrose-labelling of the tubule.

Results

Effects of temperature on toxin binding

We exposed tubules to Bti toxin at a concentration of 8 μg cm^{-3} for 60 min at temperatures of 0, 1, 2, 4, 6 and 8°C (four tubules at each temperature). At room temperature, this concentration of toxin was sufficient to kill the cells in 6–8 min, as judged by the failure of fluid secretion and staining of the cells with Trypan Blue. Tubules that had been treated with toxin at 0, 1, 2 and 4°C for 60 min, then transferred at room temperature to toxin-free saline containing Trypan Blue, secreted fluid at normal rates for more than one hour and none of the cells stained blue. However, after 60 min in toxin at 6 and 8°C, the tubules were already killed – in toxin-free saline at room temperature they secreted no fluid and the cells stained very rapidly with Trypan Blue.

Tubules treated with toxin at 8 μg cm^{-3} at 0°C for varying periods were returned to toxin-free saline at room temperature. After 20 min they were exposed to the toxin-containing saline again, but now at room temperature. The rates of fluid secretion were measured until they had declined to a low level. Tubules exposed at 0°C to 8 μg cm^{-3} of toxin for periods of 40, 60, 100 and 120 min (four tubules for each period) later secreted fluid at normal rates in toxin-free saline at room temperature but, on return to toxin-containing saline, fluid secretion failed earlier than in control tubules exposed only to 0°C for the same period (Fig. 1). Evidently some toxin had bound to the tubules at 0°C. This was confirmed by the results of exposing tubules to 8 μg cm^{-3} of toxin at 0°C

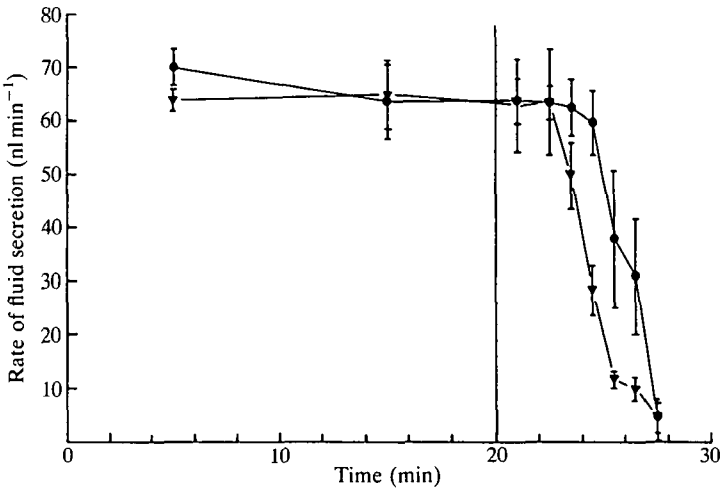

Fig. 1. Fluid secretion by tubules exposed to Bti toxin at 8 μg cm^{-3} at the time indicated by the vertical line. Some of the tubules had previously been treated with toxin for 120 min at 0°C (triangles; N=4) with control tubules from saline alone at 0°C for 120 min (filled circles; N=4). The lines attached to the mean values represent ±S.E.

for 160 and 180 min (four tubules for each period). None of these tubules secreted fluid on return to toxin-free saline at room temperature; control tubules exposed only to 0 °C for the same periods secreted fluid at high rates for at least 30 min on return to room temperature. Low temperature evidently slows toxin attachment to the tubules but does not prevent it.

Effects of pH on toxin binding

The pH of the gut lumen in species of insect susceptible to toxins from *Bacillus thuringiensis* is often high (Dow, 1986). We examined the effects of treating tubules with toxin at high pH. As a control, we examined the effects of pH alone and found that tubules were killed by exposure for 5 s to pH 11.4 (four tubules) and were irreversibly affected even by a one second exposure to this high pH (four tubules). However, they survived 40 min at pH 9 (eight tubules) and quickly recovered fully from a 10 min exposure to pH 10 (six tubules). Our tests of toxin effectiveness were, therefore, carried out at pH 10. Tubules were killed by exposure to Bti toxin at 10 $\mu g\,cm^{-3}$ for 10 min at pH 7 (N=20), but survived 10 $\mu g\,cm^{-3}$ (N=4) and 20 $\mu g\,cm^{-3}$ (N=4) for 10 min at pH 10, though were killed by a 10 min exposure to 40$\mu g\,cm^{-3}$ of Bti toxin at pH 10 (N=4). So, Malpighian tubules are less affected by the toxin at pH 10 than at pH 7.

Effects on toxin sensitivity of pretreatment with ouabain, bumetanide and 5-HT

Ouabain and bumetanide are drugs that bind to specific proteins in cell membranes, the Na/K ATPase and the Na,K,2Cl – cotransport system, respectively. We pretreated tubules for 40 min in saline containing 5-HT and one of these drugs at a concentration of 10^{-4} mol l^{-1}. We then exposed the tubules to more of the same saline but now containing 8 $\mu g\,cm^{-3}$ Bti toxin. Ouabain does not affect 5-HT-stimulated fluid secretion, so we could follow the development of toxic effects by measuring the rate of fluid secretion. Fluid secretion failed in six tubules pretreated with ouabain with a time course identical to six control tubules not first exposed to ouabain. Bumetanide greatly reduces fluid secretion rates in 5-HT-stimulated tubules. So we compared the rate at which tubules stained with Trypan Blue during toxin treatment after pretreatment for 30 min in saline containing 1.5×10^{-5} mmol l^{-1} bumetanide or in saline alone. The results showed no difference in the rates of staining between six bumetanide-treated and six control tubules.

The permeability of the tubule cell membranes to mannitol increases more than 10 times in the first 90 min of treatment with 5-HT (O'Donnell *et al.* 1984). We determined whether or not this effect on the cell membranes affected the development of toxic effects in tubules exposed to Bti toxin. Four tubules (two tubules from each of two insects) were allowed to secrete fluid for 140 min in 5-HT-containing saline. The other four tubules from the same insects were kept in 5-HT-free saline. All the tubules were then placed in saline containing 5 $\mu g\,cm^{-3}$ Bti toxin and Trypan Blue. All started to stain with Trypan Blue at similar times. The changes in the cell membrane that follow 5-HT treatment do not affect the development of toxic effects on exposure to Bti toxin.

Effects of Bti toxin on the trans-epithelial and basal cell membrane potential

We have previously reported the effects of Bti toxin on the trans-epithelial potential (TEP) of *Rhodnius* Malpighian tubules, showing that fluid secretion and the TEP are affected simultaneously (Maddrell *et al.* 1988). The TEP changes from about 40 mV lumen negative often to more than 100 mV lumen positive. Close inspection of the potential changes usually showed that the potential change occurred in two stages, the second very closely following the first (Fig. 2).

To investigate the possibility that the first change in potential might reflect a collapse in the normal potential of the basal cell membrane, we measured this with an intracellular microelectrode. The results showed clearly that the cell potential did not at first change at all from its initial value of about −60 to −70 mV but later it rapidly collapsed to zero (Fig. 3). It follows that the fractionally later positive-going element in the TEP change must occur across the luminal cell membrane.

We repeated the experiments on toxin effects on the TEP using a K-rich saline (containing 145 mmol l^{-1} K^+ and 5 mmol l^{-1} Na^+). In this saline the TEP is 20–40 mV lumen positive and, upon exposure to Bti toxin, changes

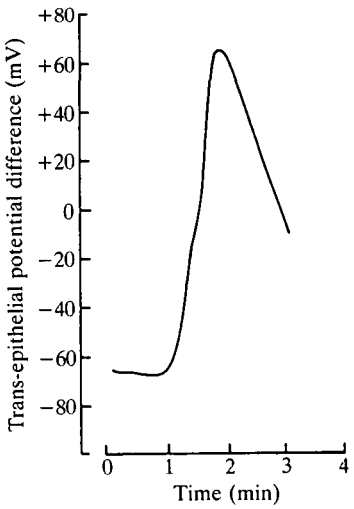

Fig. 2. The changes in trans-epithelial potential difference brought about by exposing a tubule to 60 $\mu g\,cm^{-3}$ Bti toxin in standard saline at time zero. The line was traced from the original chart recording.

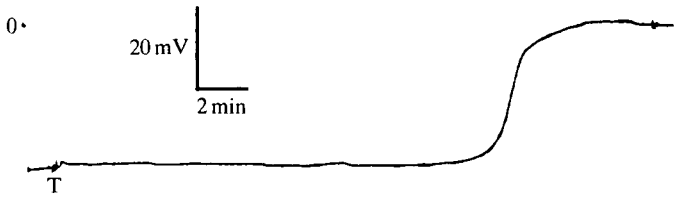

Fig. 3. Changes in basal cell membrane potential following exposure of a tubule to 2 $\mu g\,cm^{-3}$ Bti toxin (T) at time zero.

relatively little, the large positive-going step being very much reduced, though not completely removed (Fig. 4).

Sucrose loading

Sucrose, M_r 342, is normally too large to penetrate cell membranes, but might enter through toxin-induced pores. We, therefore, measured the labelling of tubules by radioactive sucrose after immersing them in saline containing Bti toxin at 15 $\mu g\,cm^{-3}$ and [^{14}C]sucrose at 20 000 cts min^{-1} μl^{-1} for different times. The numbers of radioactive counts found in the tubules at these times are given in Fig. 5 and show that as soon as its rate of fluid secretion starts to decline, more radioactive sucrose is already associated with a tubule. In fact, after 4.5 min in this toxin solution, when the rate of fluid secretion had not yet been significantly depressed, there is an indication that sucrose entry had begun; the number of radioactive counts had increased from 43 ±3 (N=8) in the first 3 min to 74±7 (N=5) after 4 min, a small but significant increase.

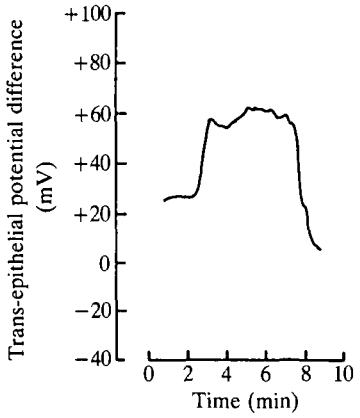

Fig. 4. The changes in trans-epithelial potential difference brought about by exposing a tubule to 20 $\mu g\,cm^{-3}$ Bti toxin in K-rich saline at time zero. The line was traced from the original chart recording.

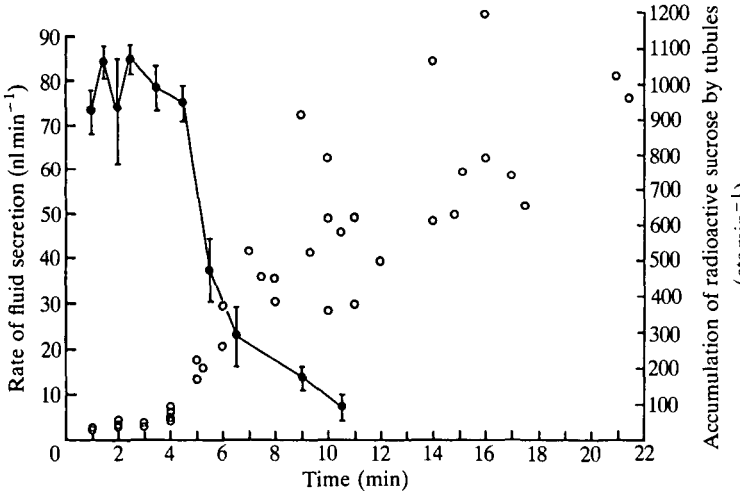

Fig. 5. The rates of fluid secretion (filled circles) and accumulation of radioactive sucrose (open circles) by Malpighian tubules immersed in saline containing 15 $\mu g\,cm^{-3}$ Bti toxin at time zero. The lines attached to the mean values represent ±S.E.

Osmotic protection experiments

If Bti toxin exerts its damaging effects by forming pores in the cell membrane, which lead in turn to the lysis of the cells by osmosis, it might be possible to reduce its effects on Malpighian tubules by adding an osmotic protectant to the bathing fluid.

Raffinose, M_r 504, has been found to protect cells in culture against the effects of Bti toxin (Knowles and Ellar, 1987). For our experiments, we exposed Malpighian tubules to Bti toxin at 1.25, 2.5, 5, 10 and 20 $\mu g\,cm^{-3}$ and included 170 mmol l^{-1} raffinose in the bathing fluid. In each case (N=20), secretion failed no more slowly than did control tubules, not exposed to raffinose, from the same insects; indeed at 1.25 and 2.5 $\mu g\,cm^{-3}$ Bti toxin, secretion failed slightly more rapidly in the presence of 170 mmol l^{-1} raffinose.

In further experiments we replaced raffinose with 60 mmol l^{-1} dextran, M_r nominally 5000. We now found that secretion failure and, particularly, staining of the cells with Trypan Blue were significantly delayed in comparison with control tubules from the same insects exposed to saline containing Bti toxin but no dextran (Fig. 6).

Experiments to minimise changes in intracellular composition

Even in tubules exposed to Bti toxin with dextran, secretion failed relatively rapidly. Perhaps osmotic imbalance is of less significance than changes in cellular concentrations of ions and metabolites. Toxin-produced pores in the cell membrane might, for example, lead to influx into the cell of calcium and magnesium ions from the bathing saline and this might disrupt cell activity.

We exposed tubules to 2.75, 3.75, 5, 7.5, 15 and 30 $\mu g\,cm^{-3}$ Bti toxin in the presence or absence of the standard 2.0 mmol l^{-1} calcium ions. There were no differences between the effects of the toxin on tubules bathed in calcium-free saline and calcium-containing saline (N=16).

As an extension of these experiments, we treated tubules with saline containing 2.75, 3.75, 5 and 7.5 $\mu g\,cm^{-3}$ Bti toxin with or without the usual concentrations of calcium and magnesium ions (2.0 and 8.5 mmol l^{-1}, respectively). Again this had no effect on the time course of failure of fluid secretion induced by the toxin (N=8).

Finally, we tried exposing tubules to Bti toxin (2.5, 5 and 10 $\mu g\,cm^{-3}$, N=12) in standard saline or in the saline whose composition had been adjusted to that typical of the intracellular milieu. This complex saline was no more successful in allowing secretion to persist under toxin action than was control saline.

Comparison of the effects of Bti toxin and melittin

We previously showed (Maddrell *et al.* 1988) that Malpighian tubules of *Rhodnius* respond to different concentrations of Bti toxin with time courses inversely related to

the dose. In any particular case, the rate of fluid secretion and/or the TEP remain unchanged for a period, but then they rapidly collapse. Fig. 7 shows a typical experiment on the effects of different concentrations of melittin on the rate of fluid secretion by isolated Malpighian tubules. Just as with Bti toxin, the delay before an effect is increased as the dose of melittin is decreased, and the rate at which secretion declines is decreased.

A more detailed comparison of Fig. 7 with results obtained with Bti toxin suggested that, once affected, fluid secretion declines somewhat more slowly with melittin than with Bti toxin. Fig. 8 shows typical results of one of several experiments to test this directly. In this case, the behaviour of two tubules exposed to 4 $\mu g\,cm^{-3}$ melittin was compared with the behaviour of two tubules exposed to 9 $\mu g\,cm^{-3}$ Bti toxin. In both cases secretion was affected within about 6 min of exposure to the toxins, but with Bti toxin secretion rates declined to below 10 $nl\,min^{-1}$ within a further 3 min, while with melittin a further 9 min was required before the secretion rate fell to below 10 $nl\,min^{-1}$.

In experiments with melittin at concentrations higher than 10 $\mu g\,cm^{-3}$, fluid secretion was affected very quickly; even when we measured the rate of fluid secretion at 30 s intervals, which is as frequent as possible, we were not able to find evidence of any delay before fluid secretion started to fail ($N=12$).

We conclude that, by comparison with Bti toxin, melittin starts to act faster but then its effects develop more slowly. In other respects their actions are similar.

We extended these experiments to include measurements of the TEP during exposure to melittin or Bti toxin. Typical results are shown in Fig. 9. The changes in TEP produced by, say, 10 $\mu g\,cm^{-3}$ Bti toxin and by 5 $\mu g\,cm^{-3}$ melittin, are very similar. There was a rapid change in potential from lumen negative to lumen positive, followed by a slower decline towards zero. However, with Bti toxin there was a delay before the

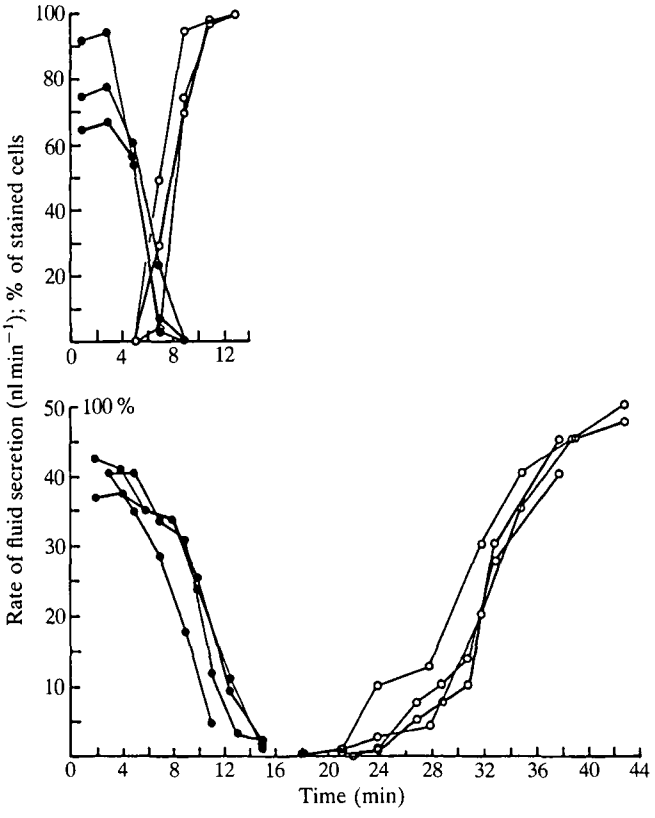

Fig. 6. The effects of 60 $mmol\,l^{-1}$ dextran in delaying the toxic effects of 20 $\mu g\,cm^{-3}$ Bti toxin. The upper panel shows the rates of fluid secretion (filled circles) and the percentage of cells stained with Trypan Blue (open circles) for three control tubules bathed in saline plus toxin only. The lower panel shows the delayed onset of failure of fluid secretion (filled circles) and the even more delayed staining with Trypan Blue (open circles) in tubules treated with toxin in saline containing 60 $mmol\,l^{-1}$ dextran. The increased osmotic concentration of the dextran-containing saline depresses the rates of fluid secretion of tubules in it.

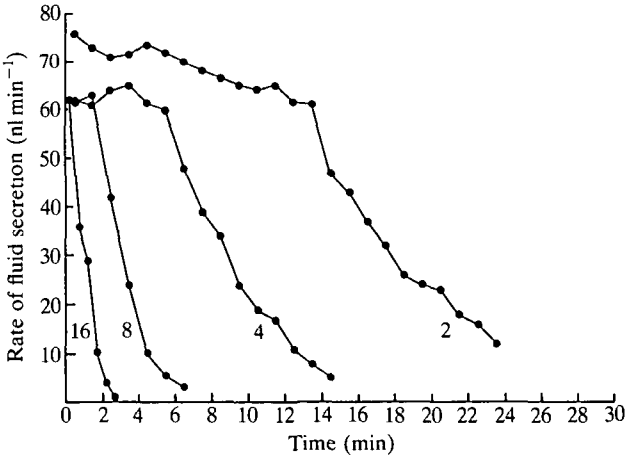

Fig. 7. The effects of different concentrations of melittin (16, 8, 4 and 2 $\mu g\,cm^{-3}$) on the rates of fluid secretion by Malpighian tubules.

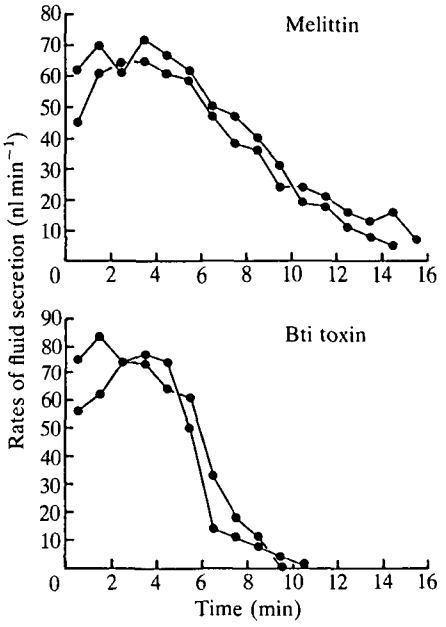

Fig. 8. A comparison of the effects of melittin (4 $\mu g\,cm^{-3}$; upper panel) and Bti toxin (9 $\mu g\,cm^{-3}$; lower panel) on fluid secretion by Malpighian tubules.

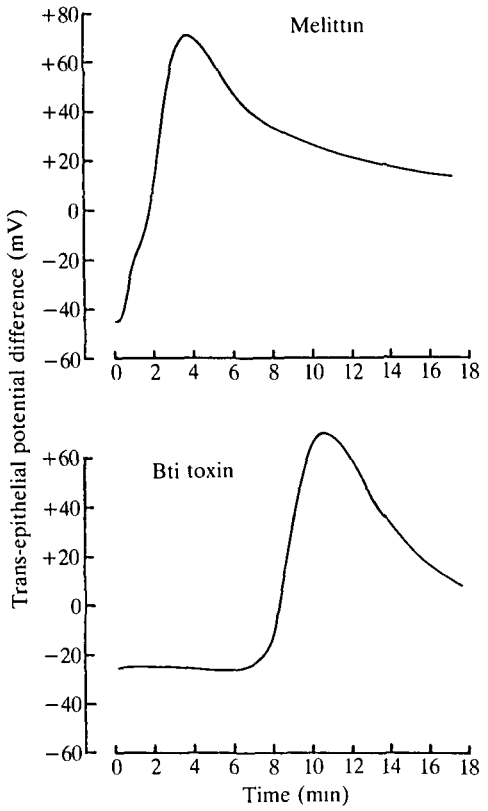

Fig. 9. A comparison of the effects of melittin (5 $\mu g\,cm^{-3}$; upper panel) and Bti toxin (10 $\mu g\,cm^{-3}$; lower panel) on the trans-epithelial potential difference of Malpighian tubules. The lines were traced from the original chart recordings.

change in TEP, but with melittin, the potential change was almost immediate. At lower concentrations of both toxins, the same difference was evident; after a delay, Bti toxin acted relatively quickly, but the effects of melittin were slower to develop.

Possible preformation of aggregates of toxin molecules

Our earlier work (Maddrell *et al.* 1988) left us with the suggestion that Bti toxin acts only when several molecules are aggregated on the cell surface. We tested the possibility that effective aggregates might form in concentrated solutions of the toxin. For this, we solubilised and activated 50 μg of the toxin in 50 μl of saline; 25 μl of this was diluted 200 times (to give a solution containing 5 $\mu g\,cm^{-3}$ of the toxin), the rest was left undiluted (and so remained at a concentration of 1000 $\mu g\,cm^{-3}$). Both solutions were then kept on ice for 4 h. Then, just prior to testing, the concentrated solution was diluted to 5 $\mu g\,cm^{-3}$ and its effect compared with that of the prediluted solution. Their effects were identical (tested on 16 tubules).

Discussion

The activated 27 000 M_r toxin from *Bacillus thuringiensis* var. *israelensis* (Bti toxin) attacks a variety of cells. It is thought to do this by first binding to specific phospholipids – phosphatidyl choline, sphingomyelin, phosphatidyl ethanolamine – provided that these lipids contain unsaturated fatty acyl substituents (Thomas and Ellar, 1983). Recently, it has been suggested that other receptors may also be involved in the initial attachment (Chilcott *et al.* 1990). The evidence suggests that the binding leads to the formation of complexes of several toxin molecules at one site (Maddrell *et al.* 1988), which then insert into the cell membrane in the form of small pores. These pores are thought to cause colloid–osmotic lysis (Knowles and Ellar, 1987), i.e. an equilibration of ions through the pore resulting in a net influx of ions and so an osmotic movement of water into the cell, cell swelling and lysis. Our present results not only provide information about how the initial binding may be affected but also give more evidence that an increased cell membrane permeability, consistent with the formation of small pores there, is a constituent part of the toxin's action. It should be emphasised that our use of Malpighian tubules does not imply that they are ever a natural target of the toxin. The rationale for using them as a model system depends on the belief that the activated toxin lyses different cells in essentially the same way. Our findings on the mechanism of action of Bti toxin on *Rhodnius*' Malpighian tubules are entirely consistent with other studies with the toxin on other target cells.

Phospholipids are thought to be readily able to diffuse laterally in cell membranes at ordinary temperatures, but their mobility is restricted by cooling. It is not surprising then that we find that Bti toxin binds to Malpighian tubule cell membranes considerably more slowly at near-freezing temperatures than at room temperatures. Binding at temperatures below 6 °C is at least ten times slower than at 20 °C.

Surprisingly, we find that Bti toxin binds to Malpighian tubule cell membranes less effectively at pH 10 than at pH 7. This is unexpected as in nature the toxin acts at the elevated pH of the gut, for example in larval mosquitoes (Dow, 1986); indeed, activation of the protoxin requires alkaline enzymic processing (Lecadet and Martouret, 1962). One possibility is that our findings result from effects on the surface charge of the tubule cells as the pH is moved away from that of their normal environment. It remains to be seen whether mosquito gut cells bind Bti toxin more, or less, effectively at neutral pH than at alkaline pH.

In that the phospholipids to which Bti toxin binds are likely to be free to diffuse laterally in cell membranes to some extent, it was puzzling to find that toxin-treated tubules show no toxic symptoms when removed into toxin-free saline even when the time of exposure to toxin was only just less than that required for damage to become evident (Maddrell *et al.* 1988). One might have expected that toxin molecules attached to phospholipids would be able to move laterally – there is good evidence that they do not detach – and form toxic aggregates. That this does not occur could be due either to the immobility of phospholipids that have toxin molecules bound to them or to the inability of toxin molecules that are attached to phospholipid molecules to form the complexes that give rise to cell damage. Conceivably, toxin

molecules might attach preferentially to phospholipids immobilised by their association with integral proteins in the cell membrane. If so, toxicity might be affected by ligands attaching to such proteins. We tested this possibility by pretreating tubules with ouabain, which attaches to membrane-bound Na/K ATPase, or with bumetanide, which binds to the Na,K,2Cl–cotransport system. Neither of these treatments affected the rate of development of symptoms when the tubules were subsequently exposed to Bti toxin. This makes it more likely then that the failure of toxic effects to appear after the tubules were treated for a short time with toxin is because toxin molecules cannot associate with each other even when attached to phospholipid molecules. Conceivably, epithelia capable of such rapid fluid, ion and solute transport, as are *Rhodnius*' Malpighian tubules, may have such a tight packing of transport proteins that membrane lipid mobility may be very much reduced. In which case, perhaps only toxin molecules from solution can attach to or bind sufficiently close to toxin molecules already bound and so lead to effective associations of toxin molecules. It is worth recalling here that we were not successful in our attempts to pre-form toxin aggregates by holding activated toxin in highly concentrated solution before testing them on isolated tubules. This is not conclusive proof that such aggregates do not form, because the solutions were diluted immediately prior to testing and any aggregates may then have rapidly disassembled before their presence could be detected. Our conclusion is that aggregation of toxin molecules occurs from solution on to the surface of the membrane rather than from the coming together of single toxin molecules already attached to the membrane.

Finally, in our attempts to affect toxicity by altering the conditions under which the toxin binds, we pretreated tubules for 2 h with 5-HT, which causes an increase of an order of magnitude in the cell membranes' permeability to mannitol (O'Donnell *et al.* 1984). This too had no effect on toxin action. Of course, if toxin molecules only associate from solution with molecules already bound, the increase in membrane fluidity, which the response to 5-HT treatment implies, may be irrelevant.

Our other major objective in this work was to test the idea that Bti toxin action depends on the formation of small pores in cell membranes – in the present case the basal, haemolymph-facing membranes of the Malpighian tubules. Our finding that sucrose, M_r 342, may well start to enter the cells just before fluid secretion begins to be affected (Fig. 5) is consistent with this idea. Much more convincing is our finding that osmotic protection with dextran, M_r 5000, much delayed the appearance of toxic effects, particularly entry into the cells of Trypan Blue, M_r 961. Since raffinose, M_r 504, does not protect the tubules at all from the action of Bti toxin, it follows that any pores must allow influx of raffinose but not Trypan Blue, i.e. their cut-off is between M_r 504 and M_r 961. The finding that dextran has a relatively smaller effect on the delay before fluid secretion fails on exposure to Bti toxin (Fig. 6) suggests that fluid secretion may be affected by the loss of small molecules from within the cell and/or influx from without. This would be from changes other than the intracellular Na/K ratio, because the rate of fluid secretion is not reduced in K-free, Na-rich saline (Maddrell, 1969) when the cells load with sodium ions and are rapidly depleted of potassium ions (Maddrell, 1978).

The rapid collapse in basal membrane potential after a relatively prolonged period when it is not affected (Fig. 3) also would be expected from our working hypothesis that toxin molecules steadily assemble into aggregates that on reaching a certain size can each insert into the membrane as a small pore. Our electrical measurements also show a dramatic, very large, positive-going change immediately following the collapse of the basal membrane potential (Fig. 2). An attractive possible explanation of this comes from the recent finding that Bti pores in lipid bilayers have high permeability to both Na^+ and K^+, but very little permeability to Cl^- (Knowles *et al.* 1989). So, pores appearing in the basal membrane would not only cause the observed collapse in the basal membrane potential (which, of course, depends on the much higher permeability to K^+ than Na^+), but would also lead to a rapid change in the Na/K ratio of the intracellular fluid, as Na^+ enters and K^+ leaves. The level of chloride ions in the cell would be much less affected. It is supposed that the cation pump on the luminal cell membrane has a higher affinity for Na than K (Maddrell, 1978). Perhaps then the elevation in the intracellular sodium concentration causes considerably faster cation transport and a consequent large increase in the apical membrane potential, without this being short-circuited by any increase in the intracellular level of Cl^-. Since the basal membrane potential is by then close to zero, the whole of the large apical membrane potential appears as the trans-epithelial potential.

Studies with the bee venom, melittin, a polypeptide composed of 26 amino acid residues, support the idea that its action depends on the formation of melittin tetramers in the membrane (Vogel and Jahnig, 1986). The hydrophilic sides of four bilayer-spanning helices face each other to form a hydrophilic pore through the membrane. It is interesting to use our mathematical model of toxin action (Maddrell *et al.* 1988) to predict how the responses of *Rhodnius* Malpighian tubules to melittin and to Bti toxin should differ. Since melittin pores depend on the formation of tetramers and if Bti pores depend on the formation of aggregates or complexes involving more, possibly several more, toxin molecules, then we would expect melittin action to develop relatively slowly once it has begun. Bti toxin action beginning at a similar time should develop faster. This conclusion, of course, depends on the proviso that the size of the pores is not markedly different in the two cases. The available evidence is consistent with this; melittin pores are permeable to raffinose, M_r 504, but not to polyethylene glycol, M_r 1000 (Tosteson *et al.* 1985), while Bti pores are also permeable to raffinose but impermeable to Trypan Blue, M_r 961 (present results) and impermeable to polyethylene glycol of M_r 1000 (Knowles and Ellar, 1987). Our results (Figs 8 and 9) show that, once the effects are discernible, they develop relatively more

quickly in the case of Bti toxin. The results with melittin were not as clear cut as with Bti toxin, especially over longer time periods. This is reminiscent of work on melittin lysis of red blood cells (Tosteson *et al.* 1985), where the effects of melittin proceed rapidly during the first few minutes but then slow and stop, perhaps because the melittin tetramers are then internalised. However, the differences in the time course of action of the two toxins when they act quickly is consistent with what is supposed about the number of molecules involved in forming a single pore.

We thank Colin Nicholls of the Department of Biochemistry for purifying the 27 000 M_r Bti toxin.

References

BURGESS, G. M., MCKINNEY, J. S., FABIATO, A., LESLIE, B. A. AND PUTNEY, J. W. (1983). Calcium pools in saponin-permeabilized guinea pig hepatocytes. *J. biol. Chem.* **258**, 15 336–15 345.

CHILCOTT, C. N., KNOWLES, B. H., ELLAR, D. J. AND DROBNIEWSKI, F. A. (1990). Mechanism of action of B.t.i. parasporal body. In *Bacterial Control of Mosquitoes and Blackflies* (ed. H. De Barjac and D. Sutherland). Rutgers University Press, New Jersey (in press).

DAVIDSON, E. W. AND YAMAMOTO, T. (1984). Isolation and assay of the toxic component from the crystals of *Bacillus thuringiensis* var. *israelensis*. *Curr. Microbiol.* **11**, 171–174.

DOW, J. A. T. (1986). Insect midgut function. *Adv. Insect Physiol.* **19**, 187–328.

KNOWLES, B. H., BLATT, M. R., TESTER, M., HORSNELL, J. M., CARROLL, J., MENESTRINA, G. AND ELLAR, D. J. (1989). A cytoloytic δ-endotoxin from *Bacillus thuringiensis* var. *israelensis* forms cation selective channels in planar lipid bilayers. *FEBS Lett.* **244**, 259–262.

KNOWLES, B. H. AND ELLAR, D. J. (1987). Colloid-osmotic lysis is a general feature of the mechanism of action of *Bacillus thuringiensis* δ-endotoxins with different insect specificity. *Biochim. biophys. Acta* **924**, 509–518.

KNOWLES, B. H., THOMAS, W. E. AND ELLAR, D. J. (1984). Lectin-like binding of *Bacillus thuringiensis* var. *kurstaki* lepidopteran-specific toxin is an initial step in insecticidal action. *FEBS Lett.* **168**, 197–202.

LECADET, M. AND MARTOURET, D. (1962). La toxine figuree de *Bacillus thuringiensis*. Production enzymatique de substances soluble toxiques par injection. *C. r. hebd. Séanc. Acad. Sci., Paris* **254**, 2457–2459.

MADDRELL, S. H. P. (1969). Secretion by the Malpighian tubules of *Rhodnius*. The movements of ions and water. *J. exp. Biol.* **51**, 71–97.

MADDRELL, S. H. P. (1978). Transport across insect excretory epithelia. In *Membrane Transport in Biology* (ed. G. Giebisch, D. C. Tosteson and H. H. Ussing). Springer: Heidelberg, New York.

MADDRELL, S. H. P. (1980). Characteristics of epithelial transport in insect Malpighian tubules. *Curr. Top. Membr. Transport*, vol. 14, pp. 428–463. New York, London: Academic Press.

MADDRELL, S. H. P., LANE, N. J., HARRISON, J. B., OVERTON, J. A. AND MORETON, R. B. (1988). The initial stages in the action of an insecticidal δ-endotoxin of *Bacillus thuringiensis* var. *israelensis* on the epithelial cells of the Malpighian tubules of the insect, *Rhodnius prolixus*. *J. Cell Sci.* **90**, 131–144.

MADDRELL, S. H. P., PILCHER, D. E. M. AND GARDINER, B. O. C. (1971). Pharmacology of the Malpighian tubules of *Rhodnius* and *Carausius*: the structure–activity relationship of tryptamine analogues and the role of cyclic AMP. *J. exp. Biol.* **54**, 779–804.

O'DONNELL, M. J. AND MADDRELL, S. H. P. (1984). Secretion by the Malpighian tubules of *Rhodnius prolixus*: electrical events. *J. exp. Biol.* **110**, 275–290.

O'DONNELL, M. J., MADDRELL, S. H. P. AND GARDINER, B. O. C. (1984). Passage of solutes through walls of Malpighian tubules of *Rhodnius* by paracellular and transcellular routes. *Am. J. Physiol.* **246**, R759–R769.

THOMAS, W. E. AND ELLAR, D. J. (1983). Mechanism of action of *Bacillus thuringiensis* var. *israelensis* insecticidal δ-endotoxin. *FEBS Lett.* **154**, 362–368.

TOSTESON, M. T., HOLMES, S. J., RAZIN, M. AND TOSTESON, D. C. (1985). Melittin lysis of red cells. *J. Membr. Biol.* **87**, 35–44.

VOGEL, H. AND JAHNIG, F. (1986). The structure of melittin in membranes. *Biophys. J.* **50**, 573–582.

WARD, E. S., RIDLEY, A. R., ELLAR, D. J. AND TODD, J. A. (1986). *Bacillus thuringiensis* var. *israelensis* δ-endotoxin. Cloning and expression of the toxin in sporogenic and asporogenic strains of *Bacillus subtilis*. *J. molec. Biol.* **191**, 13–22.

(Received 17 May 1989 – Accepted 22 July 1989)