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CHARACTERIZATION OF MEMBRANE PERMEABILITY ALTERATIONS INDUCED IN VERO CELLS BY *CLOSTRIDIUM PERFRINGENS* ENTEROTOXIN

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Summary

Alterations in plasma membrane permeability induced by *Clostridium perfringens* enterotoxin were studied using Vero (African green monkey kidney) cells which were radioactively labeled with four markers of different molecular size. The markers were α-amino[^{14}C]isobutyric acid (M_r 103), ^{3}H-labeled nucleotide (M_r approx. 300), ^{51}Cr label (M_r approx. 3000) and [^{3}H]RNA ($M_r > 25\,000$). Over a 2 h period, enterotoxin caused significant release of aminoisobutyric acid, nucleotides and ^{51}Cr label but not RNA. The effects of enterotoxin on label release were dose- and time-dependent. The rate of release of markers was dependent upon their size. Permeability alterations could be detected within 15 min with a high dose of enterotoxin. Gel chromatography of released material was used to determine that markers of M_r 3000 but not 25 000 leaked from permeabilized cells. It was concluded that enterotoxin is producing functional 'holes' of limited size in the membrane. Permeability changes due to enterotoxin treatment differed between confluent and non-confluent (growing) cells. We propose that the primary action of the enterotoxin is to interact with the plasma membrane and produce functional 'holes' of defined size. The resultant alterations in membrane permeability cause the loss of essential cellular substances which inhibits processes such as macromolecular synthesis and eventually leads to cell deterioration and death.

Introduction

Clostridium perfringens enterotoxin has been shown to induce transport alterations [1—3], metabolic disturbances [3,4], histological damage [5,6]

and inhibition of macromolecular synthesis [7] in various model systems. Recent studies [7] have demonstrated that cultured Vero (African green monkey kidney) cells are an excellent model system for studies of the mode of action of the enterotoxin. The enterotoxin causes rapid (within 30 min) inhibition of macromolecular synthesis, loss of viability and morphological alterations in these cells. Additional studies [8] have shown that radioactively labeled enterotoxin binds to Vero cells and that binding is essential for eliciting biological effects.

C. perfringens enterotoxin induces membrane blebs in intestinal epithelial cells [6] and Vero cells [7]. It has been proposed [6,7] that the mode of action of the enterotoxin involves a direct plasma membrane interaction which causes structural and functional alterations in the plasma membranes of sensitive cells. If this hypothesis is confirmed, *C. perfringens* enterotoxin could prove to be a valuable tool for studies of plasma membrane structure-function relationships.

Thelestam and Möllby have developed a sensitive assay [9—12] for the measurement of toxin-induced damage to the plasma membrane. Such damage in cultured cells can be detected by measuring leakage of cytoplasmic material into the culture medium. Furthermore, by preloading cultured cells with labeled substances of differing molecular sizes, it is possible to determine the size limits of the functional 'holes' produced by treatment with a membrane active agent. This assay system has been utilized in our studies to characterize permeability alterations induced in cultured Vero cells by *C. perfringens* enterotoxin.

Materials and Methods

Toxic substances. C. Perfringens enterotoxin was prepared as described [13] and the biological activity (erythemal units, EU) determined by methods previously reported [14]. The specific activity of the enterotoxin preparations varied from 1500—2000 EU/mg protein. Enterotoxin was dissolved in double-distilled water and 60-μl aliquots were stored frozen at —20° C until used. RNAase, protease and phospholipase C activity [17] were not detectable in the enterotoxin preparations which were determined to be greater than 99% in purity [13,15,16]. The enterotoxin has been shown to have no hemolytic activity and does not cause leakage of preloaded [^{3}H]uridine from red cells (McDonel, J.L., unpublished data). Melittin was purchased from Sigma Chemical Co.

Culture of Vero cells. Vero cells were grown in 75-cm^2 tissue culture flasks (Corning) with Medium 199 supplemented with 5% fetal calf serum (Grand Island Biological Company) and 0.75% sodium bicarbonate. When monolayers were confluent they were removed by trypsinization with 0.25% trypsin in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salts solution. Unless otherwise specified, cells to be used for leakage assays were inoculated into 16-mm wells in tissue culture cluster dishes (Costar). The seeding density was $5 \cdot 10^4$ cells in 3 ml of medium per well. The cluster dishes were incubated at 37° C in a humidified atmosphere containing 5% CO_2 in air. Fresh medium was added to the cultures every 3 days. After 6 days confluent monolayers developed which contained $6—8 \cdot 10^5$ cells per culture well.

Labeling of cells

Cells were labeled, with modifications, by the methods of Thelestam and Möllby [9—11].

RNA label. 24 h after inoculation, the cultures were washed with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salts solution (buffer) and labeled by the addition of 2 ml of Medium 199 containing 2 μCi of [^{3}H]uridine (41.3 Ci/mmol, New England Nuclear). After 24 h, the medium containing the label was removed, the cells were washed with buffer, and incubation was continued for 4 days in nonlabeled Medium 199.

^{51}Cr label. Confluent monolayers were rinsed with buffer and incubated for 1 h with 1 ml of buffer containing $Na_2{}^{51}CrO_4$ (246 mCi/mg, New England Nuclear) at a concentration of 50 μCi/ml.

Nucleotide label. Confluent, 6-day-old monolayers were washed with buffer and labeled by the addition of 2 ml of Medium 199 containing 2 μCi of [^{3}H]-uridine. After 2 h the labeling medium was removed.

α-Amino[^{14}C]isobutyric acid label. Incubation of confluent monolayers in buffer for 30 min prior to labeling was found to optimize aminoisobutyric acid uptake with minimal spontaneous release of label. Following removal of the buffer, the monolayers were labeled for 1 h by the addition of 1 ml of Medium 199 containing 1 μCi of amino[^{14}C]isobutyric acid (51.6 mCi/mmol, New England Nuclear). The monolayers were then washed with Medium 199 for 30 min to decrease the rate of spontaneous release.

Confluent vs. nonconfluent (growing) cultures. Confluent cultures were labeled as described above for nucleotides and RNA. To obtain a growing cell population, freshly trypsinized cells were inoculated into 35-mm culture dishes (Falcon) at a concentration of $2.5 \cdot 10^5$ cells per dish. After 24 h there were $4—5 \cdot 10^5$ cells per dish. For RNA-labeling of growing cells, 2 ml of Medium 199 containing [^{3}H]uridine (1 μCi/ml) were added to each culture at inoculation, followed by incubation for 20 h. The labeling medium was then removed and the cells were incubated for 4 h in nonlabeled Medium 199. To obtain growing cell cultures (with nucleotide label), 2 ml of Medium 199 containing [^{3}H]uridine (1 μCi/ml) were added to 22-h monolayers. After 2 h the labeling medium was removed.

Maximal release. The maximal release of cytoplasmic label was determined after cell membrane rupture by the addition of 1 ml of 1 M citric acid [18] and 1 ml of a 0.5% saponin buffer [19] per well. This procedure rapidly lysed plasma membranes and yielded intact nuclei as determined by light microscopy and leakage rate studies of acid-insoluble material from cells labeled (prior to rupture) with [^{3}H]thymidine.

After 15 min of cytoplasmic membrane rupture with citric acid/saponin the nuclei were removed by centrifugation at 4°C for 10 min at 1000 × *g*. Radioactive label was counted from the supernatant (also referred to as the 'cell lysate') [9]. The total value for maximal release from confluent cultures was $4—5 \cdot 10^5$ cpm/culture for RNA and ^{51}Cr label, $1—2 \cdot 10^5$ cpm/culture for nucleotide label and $2—4 \cdot 10^4$ cpm/culture for aminoisobutyric acid label. Maximal release from growing cultures was $4—5 \cdot 10^5$ cpm/culture for RNA label and $2—3 \cdot 10^5$ cpm/culture for nucleotide label.

Spontaneous release. Spontaneous release after 30 min at 37°C was less than

1% for RNA label, 3% for nucleotide label, 10% for ^{51}Cr label and 30—40% for aminoisobutyric acid label.

Measurement of radioactivity. Samples (0.2 ml) from ^{3}H- and ^{14}C-labeled cultures were counted in a Packard Tricarb liquid scintillation spectrophotometer. ^{51}Cr samples (0.5 ml) were counted in a Packard gamma spectrophotometer.

Treatment of cells with toxins. All cultures were washed with buffer or Medium 199 without serum prior to these treatments. Radioactively labeled cultures were incubated with the test substance diluted in 2 ml of Medium 199 without serum. Following the desired incubation time, 0.5 ml of culture supernatant was gently removed and centrifuged at 4° C for 3 min in an Eppendorf microcentrifuge. Radioactivity was measured in the supernatant. Released radioactivity was calculated as described by Thelestam and Möllby [11]:

$$\% \text{ of maximal release} = \frac{\text{toxin-induced release—spontaneous release}}{\text{maximal release—spontaneous release}} \times 100$$

All experiments were performed in duplicate or triplicate.

Estimate of size of labeled material

Perchloric acid precipitation. Carrier yeast RNA (200 μg) was added to 1 ml of cell lysate. Acid-insoluble material in this sample was precipitated by the addition of 0.5 ml of cold (4° C) 25% perchloric acid containing 0.75% uranyl acetate [9]. The precipitate was then centrifuged at 4° C for 10 min at 2000 × *g* and hydrolyzed at 90° C for 30 min in 25% perchloric acid. Counts in the hydrolyzed precipitate and supernate represented RNA and acid-soluble nucleotides, respectively.

Gel chromatography. The molecular size of cell lysate and leaked material from RNA- and nucleotide-labeled cultures was determined by chromatography on a Biogel P-60 (Bio-Rad Laboratories) column [9]. Biogel P-60 was equilibrated in 10 mM Tris-HCl, 140 mM LiCl and 1 mM $MgCl_2$, pH 7.6. The column was 2.5 × 120 cm and the flow rate was 3 ml/h per cm^2. Fractions (6-ml) were collected and radioactivity was determined from 1 ml samples of each fraction by liquid scintillation spectrophotometry.

Cell lysates and leaked material from aminoisobutyric acid-labeled cultures were chromatographed on a Biogel P-2 (Bio-Rad Laboratories) column [10]. Biogel P-2 was equilibrated in Tris-buffered saline which contained 0.02 M Tris-HCl and 0.15 M NaCl, pH 7.0. The column was 2.5 × 24.5 cm and the flow rate was 2.7 ml/h per cm^2. Fractions (4.5 ml) were collected and radioactivity in a 0.2 ml sample was determined from each fraction.

Gel chromatography of ^{51}Cr-labeled material was performed on a Sephadex G-25 (Pharmacia) column [11] which was equilibrated in Tris-buffered saline. The column was 2.5 × 50 cm and the flow rate was 2.7 ml/h per cm^2. Radioactivity was determined in 3-ml aliquots from each 4.5 ml fraction.

Results

Labeling conditions

Thelestam and Möllby [9] have reported a protocol, using [^{3}H]uridine, for

differential labeling of early growth or stationary phase human diploid fribroblasts. This protocol resulted in cultures which contained labeled cytoplasmic material of predominately high or low molecular weight, referred to as RNA- and nucleotide-labeled cultures, respectively.

To confirm that similar differential labeling conditions could be obtained with Vero cells, perchloric acid precipitations and gel chromatography were performed on cell lysates from RNA- and nucleotide-labeled Vero cultures. In lysate from RNA-labeled cultures, 90% of the counts were acid-insoluble while in lysate from nucleotide-labeled cultures less than 30% were acid-insoluble.

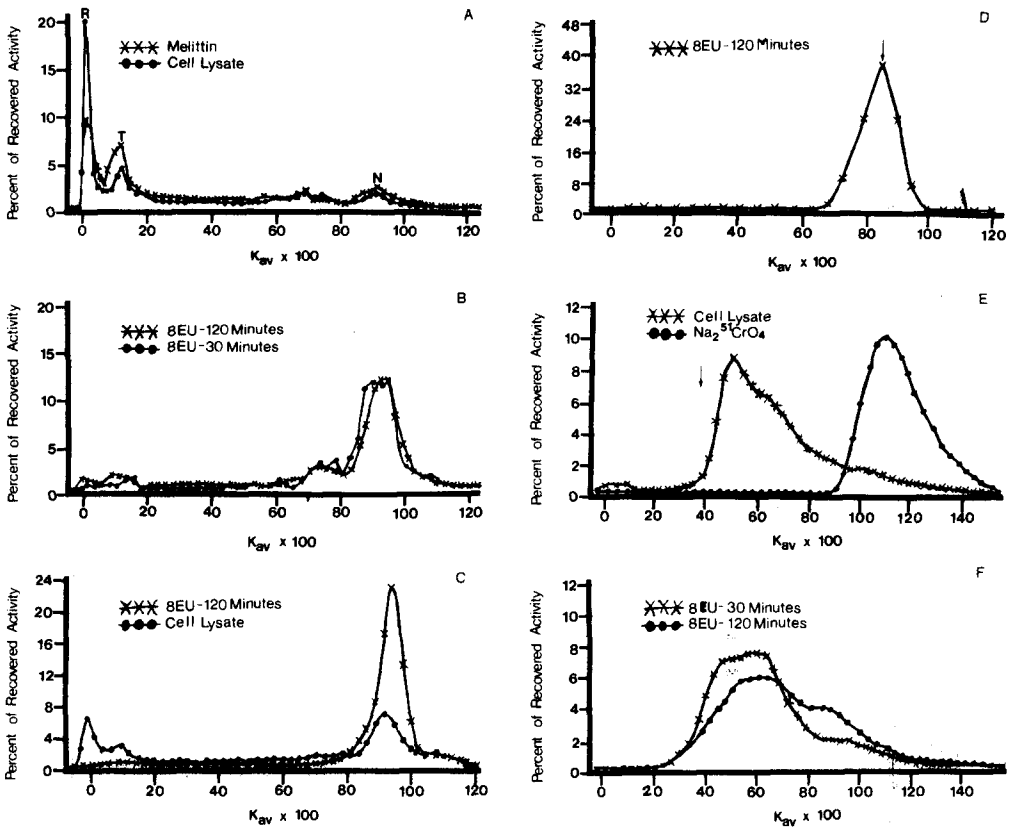

Fig. 1. Gel chromatography of cell lysates and material released by toxic substances. (A) Biogel P-60 chromatography of RNA-labeled cell lysate (●———●) material released by a 120 min treatment of RNA-labeled cultures with melittin (x———x). (B) Biogel P-60 chromatography of released material from enterotoxin-treated, RNA-labeled cultures. Treatment with 8 EU of enterotoxin for 30 (●———●) and 120 (x———x) min. (C) Biogel P-60 chromatography of nucleotide-labeled cell lysate (●———●) and material released by a 120 min treatment of nucleotide-labeled cultures with 8 EU of enterotoxin (x———x). (D) Biogel P-2 chromatography of material released by a 120 min treatment of aminoisobutyric acid-labeled cultures with 8EU of enterotoxin. The arrow indicates the position of the peak when aminoisobutyric acid or cell lysate from aminoisobutyric acid-labeled cultures was chromatographed. (E) Sephadex G-25 chromatography of ^{51}Cr-labeled cell lysate (x———x) and $Na_2{}^{51}CrO_4$ (●———●). Arrow shows the position of the peak obtained when glucagon (M_r about 3500) was chromatographed. (F) Sephadex G-25 chromatography of material released by enterotoxin treatment of ^{51}Cr-labeled cells. The effects of 8 EU of enterotoxin treatment for 30 (x———x) and 120 (●———●) min are shown.

Following chromatography, radioactivity in each fraction was measured, background activity was subtracted from each value and data were expressed as percentage of total recovered activity.

When lysate from an RNA-labeled culture was chromatographed on a Biogel P-60 column (Fig. 1A), two high molecular weights peaks predominated. The first peak coincided with the elution profile of ribosomal RNA ($M_r > 200\,000$), while the second coincided with that of transfer RNA (M_r 25 000). Fig. 1C shows that three large peaks appeared when lysates from nucleotide-labeled cultures were applied to the Biogel P-60 column. These peaks are referred to as the rRNA, tRNA and nucleotide peaks [9]. Over 60% of the radioactivity recovered from the nucleotide-labeled lysates was in a single low molecular weight peak of which the elution profile coincided with that of uridine. Collectively, these results confirm that differential labeling of the Vero cells was achieved.

^{51}Cr-labeled cell lysate has been reported [11] to elute on Sephadex G-25 as a heterogeneous peak of M_r approx. 3000. Similar results were obtained in our studies (Fig. 1E). Some cell cultures were labeled with the non-metabolizable amino acid, amino[^{14}C]isobutyric acid (M_r 103). Aminoisobutyric acid-labeled cell lysate eluted on Biogel P-2 as a single peak (not shown) which coincided with the elution profile of aminoisobutyric acid (Fig. 1D). This confirmed that aminoisobutyric acid is not being metabolized by the Vero cells. The data from the elution profiles indicate the following order in size of the markers used in this study: RNA label ($M_r > 25\,000$) > ^{51}Cr label (M_r about 3000) > nucleotide label (M_r about 300) > aminoisobutyric acid label (M_r 103). Comparable labeling conditions were achieved for RNA- and nucleotide-labeling of confluent and growing cell cultures.

Morphological effects of toxic substances

Confluent Vero cell cultures treated with enterotoxin exhibited morphological alterations which were visible under the light microscope. Following treatment with enterotoxin, there was an initial change from the spindle shape characteristic of normal Vero cells to a spherical form. With longer periods of incubation with enterotoxin, the rounded cells aggregated. Even at high doses, enterotoxin-induced cell detachment was minimal (less then 10%). This contrasts with earlier studies with nonconfluent (growing) Vero cultures [7], where extensive detachment was noted. The onset of morphological alterations was dose-dependent. When 8 EU (2 μg/ml) of enterotoxin were added to these cultures, cytopathological effects were evident within 15 min. Following treatment with 1 EU (0.25 μg/ml) damage was not apparent until after 45 min. At a concentration of 10 μg/ml, melittin caused morphological alterations in the confluent cultures with between 15 and 30 min of treatment.

Enterotoxin-induced release of aminoisobutyric acid label from confluent cultures

Treatment of confluent cultures with *C. perfringens* enterotoxin caused a rapid release of aminoisobutyric acid label (Fig. 2). Nearly 100% of the aminoisobutyric acid label was released by 8 EU after 30 min of enterotoxin treatment. The leakage effect of enterotoxin was dose- and time-dependent. When high doses of enterotoxin (8 EU) were applied to the culture, the most rapid leakage occurred during the initial 10 min of treatment. With low doses of enterotoxin (2 EU), substantial leakage was not detectable during the first 10

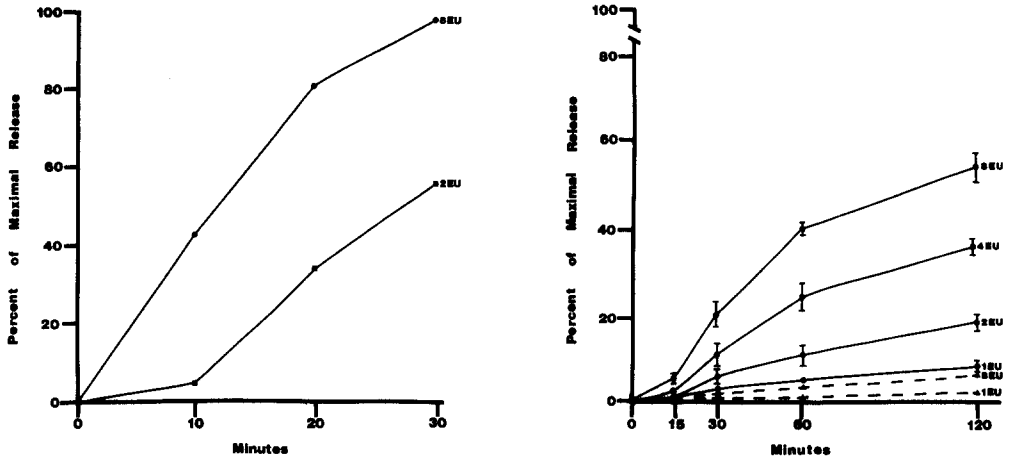

Fig. 2. Effects of enterotoxin on release of aminoisobutyric acid label. The effects of a high (8 EU) and low (2 EU) dose of enterotoxin on release of aminoisobutyric acid label are shown.

Fig. 3. Effects of enterotoxin on release of nucleotide label (———) and RNA label (- - - - -). Bars represent the standard error of the mean for each point. Points without bars had standard errors too small to depict. The results shown are from triplicate experiments.

min of treatment but increased rapidly after 10—20 min of treatment. Released material from enterotoxin-treated cultures was run on a Biogel P-2 column (Fig. 1D). This material had an elution profile which coincided with that of aminoisobutyric acid and cell lysate from aminoisobutyric acid-labeled cultures.

Enterotoxin-induced release of nucleotide label from confluent cultures

C. perfringens enterotoxin also induced leakage [8] of nucleotide label (Fig. 3). Over 50% of the cytoplasmic label in these cells was released by 8 EU within 2 h. The leakage effect of enterotoxin was dose- and time-dependent. At all enterotoxin doses, leakage was most rapid between 15 and 30 min after exposure. Released material from cultures treated with 8 EU of enterotoxin for 120 min was run on a Biogel P-60 column (Fig. 1C). The peak recovered had an elution profile which coincided with that of nucleotides from cell lysate. Similar chromatographic results were obtained with leaked material from cultures treated with 8 EU for only 30 min (data not shown). These results indicate that primarily low molecular weight material was leaking from treated cells. The chromatographic results, which were similar with 30 and 120 min of enterotoxin treatment, suggest that the size of the functional 'holes' was not grossly enlarging with time. It should be noted that the term 'hole' is used as an operational term [11] and does not necessarily refer to a fixed physical entity in the membrane.

Enterotoxin-induced release of ^{51}Cr label from confluent cultures

Fig. 4 shows that enterotoxin caused the release of ^{51}Cr label. About 50% of the cytoplasmic ^{51}Cr label was released within 2 h by 8 EU. The effects of enterotoxin on ^{51}Cr label leakage were dose- and time-dependent. With an

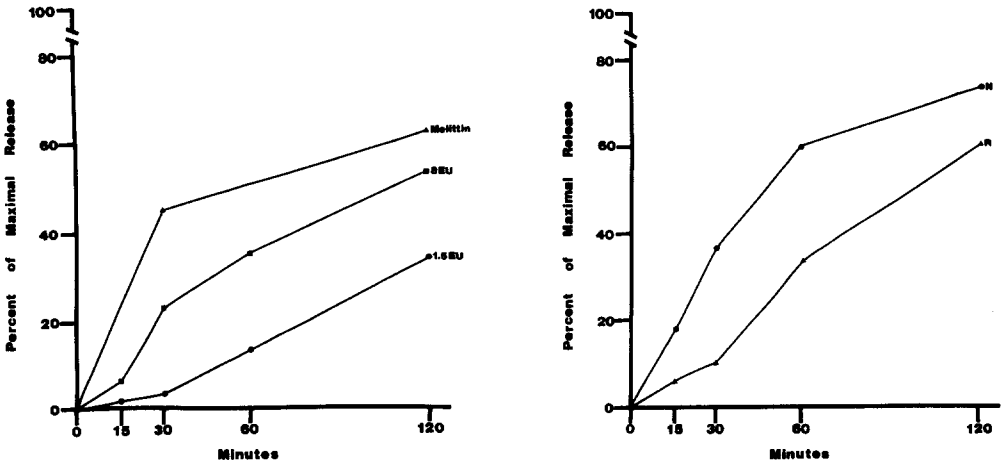

Fig. 4. Effects of enterotoxin and melittin on ^{51}Cr-labeled cells. The effects of 8 EU and 1.5 EU of enterotoxin and 10 μg/ml of melittin on release of ^{51}Cr label are shown.

Fig. 5. Effects of melittin on release of nucleotide label (●———●) or RNA label (▲———▲) cultures. Melittin was used at a concentration of 10 μg/ml.

enterotoxin dose of 8 EU, leakage was most rapid between 15 and 30 min after treatment. Melittin (10 μg/ml) caused a rapid release of ^{51}Cr label as previously reported [11]. Samples of released material from cultures treated with enterotoxin for 30 or 120 min were chromatographed on a Sephadex G-25 column (Fig. 1F). The results obtained showed heterogeneous peaks with elution profiles similar to those of cell lysate.

Enterotoxin-induced release of RNA label from confluent cultures

C. perfringens enterotoxin induced little leakage from RNA-labeled cultures (Fig. 3). When these cultures were treated with 8 EU for 120 min, they released less than 10% of maximal release. Released material from these cultures chromatographed on Biogel P-60 as a single peak (Fig. 1B) with an elution profile which coincided with the nucleotide peak of cell lysate. Fig. 1B also shows that there was no difference in the elution profiles of leaked material from cultures treated with 8 EU for 30 or 120 min. These results strongly suggest that the leakage which occurred from RNA-labeled cells was from the small pool of labeled nucleotides present in these cells. Enterotoxin caused minimal leakage of either rRNA or tRNA. The small amounts of RNA observed chromatographically could have come from minimal enterotoxin-induced cell lysis.

Melittin has been reported [9] to cause large functional holes in treated cells. In these studies rRNA, tRNA and nucleotides leaked from melittin-treated cells. To insure that our system was sensitive to RNA leakage, melittin was used as a positive control. Fig. 5 shows that melittin (10 μg/ml) caused large amounts of leakage from both nucleotide- and RNA-labeled cultures. The released material from RNA-labeled cultures treated with melittin for 120 min was chromatographed on a Biogel P-60 column. Fig. 1A shows clearly that melittin caused leakage of rRNA, tRNA and nucleotides. Similar results were obtained from nucleotide-labeled cultures treated with melittin (not shown).

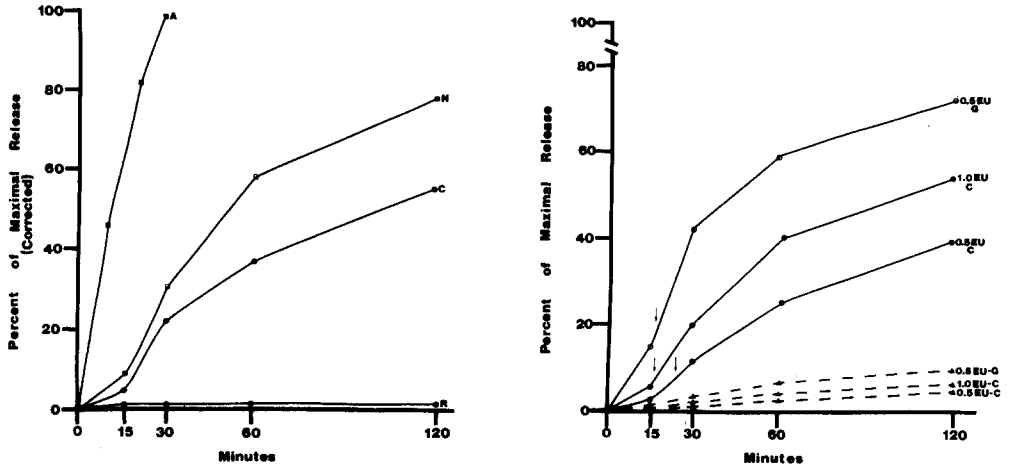

Fig. 6. A comparison of the release of materials of different molecular sizes. The release of various materials after treatment with 8 EU is shown. The results show the release of aminoisobutyric acid (■———■), nucleotides (□———□), ^{51}Cr label (●———●) and RNA (○———○). As described in the text, the release from nucleotide- and RNA labeled cells is corrected here to reflect the actual release of cytoplasmic nucleotides and RNA from enterotoxin-treated cells. The aminoisobutyric acid and ^{51}Cr label data are the same as used in Figs. 2 and 4.

Fig. 7. Differential effects of enterotoxin on growing and confluent Vero cells. The enterotoxin-induced release of material from confluent nucleotide-labeled cultures (●———●), growing nucleotide-labeled cultures (○———○), confluent RNA-labeled cultures (▲———▲) and growing RNA-labeled cultures (△———△) is shown. The doses of enterotoxin shown represent the dose of enterotoxin added per 10^5 cells. The arrows show the onset of morphological damage.

Comparison of enterotoxin-induced leakage of various markers

The formula [11] used to calculate the percent of maximal release caused by enterotoxin is based on counts per minute released as a percent of total cytoplasmic counts (nucleotide and RNA). In Fig. 6 nucleotide leakage data are expressed as a percent of the total intracellular RNA pool. Nearly 100% of maximal release from aminoisobutyric acid- and ^{51}Cr-labeled cultures was due to these labels only. Therefore correction of these data is not necessary. Using the corrected data, Fig. 6 clearly shows that enterotoxin-induced leakage was a function of the size of the label. Small labels were released most rapidly from treated cells while large labels (greater than 25 000) were not released at all. Following enterotoxin treatment, the upper limit for molecular size of leaked materials was between M_r 3000 and 25 000.

Leakage effects of enterotoxin on confluent vs. nonconfluent (growing) cells

As mentioned previously, confluent cells treated with enterotoxin rounded up but did not detach. Additionally, membrane blebs formed slowly in treated confluent cultures. They were apparent after 1 h of treatment with 4 EU of enterotoxin. In previous studies [7], cell detachment and rapid (within 30 min) membrane bleb formation were noted in nonconfluent cultures. For the current studies an investigation was made to determine if growing and confluent cells had different responses to enterotoxin. The enterotoxin-induced leakage of nucleotide and RNA label from growing and confluent cultures was studied. Fig. 7 shows that growing cultures leaked more nucleotide label (and

RNA label) than did confluent cultures. Leakage of RNA label remained minimal from the growing cultures. After 90 min of enterotoxin treatment some cell lysis in the growing cultures was noted. Collectively, these results suggest that the greater leakage from growing cells was not a result of increased size of the functional hole. Additionally, these results indicate that there was a significant correlation between onset of morphological alterations, detachment and leakage.

Discussion

Permeability alterations often are used as an index for studies on plasma membrane damage [9,20,21]. Leakage studies can yield both qualitative and quantitative information on membrane lesions. Such data are often valuable for generalized membrane studies as well as determinations of specific modes of action of toxic substances. Membrane lesions induced by many toxic substances have been previously characterized [9—11] by the use of assay procedures similar to those in this report. Membrane lesions can be classified by their leakage patterns [11]. Triton X-100 caused simultaneous leakage of all markers (from aminoisobutyric acid to RNA) and has been classified as producing large functional holes in plasma membranes [11]. Small lesions are produced by agents such as amphotericin B which caused much greater leakage of aminoisobutyric acid label than the other markers [11]. Agents such as *C. perfringens* θ toxin produced lesions of an intermediate type where two or more labels were extensively leaked from treated cells [11]. *C. perfringens* enterotoxin induces definable characteristic membrane lesions. The rate of release of the four markers from enterotoxin-treated cells was clearly size-dependent. The closest comparable lesion induced by a characterized toxic substance is the lesion induced by a low dose of θ toxin [11]. Both θ toxin (at low doses) and enterotoxin cause significant leakage of aminoisobutyric acid, nucleotide and ^{51}Cr label but not RNA. However, moderate doses of θ toxin (a dose sufficient to cause morphological damage after 30 min treatment) cause significant RNA leakage. Gel chromatography has shown that θ toxin induces tRNA leakage [9]. In contrast, doses of enterotoxin which produce morphological alterations did not induce significant tRNA or rRNA leakage. The functional holes produced by θ toxin and enterotoxin do not appear identical by these comparisons. θ toxin interacts with membrane cholesterol and causes formation of arc-shaped structures in membranes [22]. To study further the possible similarities between θ toxin and enterotoxin, electron microscopic investigations of enterotoxin-treated Vero cells are in progress.

C. perfringens enterotoxin causes a rapid leakage of aminoisobutyric acid which is followed by slower leakage of nucleotides and ^{51}Cr label. There are at least two possible explanations for this. The enterotoxin could initially induce small holes which enlarge with time. Alternatively, there could be a more rapid diffusion of aminoisobutyric acid through functional holes which are also large enough for nucleotides and ^{51}Cr to pass through. Clearly, the functional holes are not enlarging so much as to allow leakage of material greater than M_r 25 000 but a small increase in functional hole size with time is possible. The initial lag in leakage of nucleotides and ^{51}Cr label could reflect

a need for the enterotoxin-induced lesion to become larger before leakage of these labels is possible.

Hypotonic conditions stretch cell membranes and induce a selective loss of small molecules [11]. It is possible, though largely speculative, that enterotoxin could act similarly to Staphylococcal α toxin [23] by disrupting the cell's osmotic barrier. At higher concentrations, α toxin induces leakage of aminoisobutyric acid and nucleotides in a pattern [10] which is very similar to that caused by enterotoxin. Large membrane blebs form more rapidly in growing cells treated with enterotoxin than in confluent cells. As reported, growing cells treated with enterotoxin leak far more nucleotides than do confluent cells. These observations might indicate a correlation between membrane blebbing or stretching, and leakage. Because indirect conclusions are made from such data, further studies are planned to determine if enterotoxin acts to disrupt the osmotic barrier of the cell.

A number of possible explanations can be offered as to why growing cells are more susceptible to enterotoxin than are confluent cells. Since *C. perfringens* enterotoxin binds to Vero cells [8], it is possible that growing cells have more available binding sites than do confluent cells. A second possible explanation is that enterotoxin more strongly affects growing cells due to some internal metabolic difference(s) between growing and resting cells. Lastly, and perhaps most intriguing, the possibility exists that confluent cells can better repair enterotoxin-induced membrane lesions. Non-growing L-fibroblasts are known to have a greater membrane repair capacity than dividing cells [24]. When the nature of the enterotoxin-induced lesion becomes better understood, enterotoxin might potentially be useful as a tool for studying membrane repair processes.

A previous study using growing cells reported [7] that within 30 min *C. perfringens* enterotoxin totally inhibited incorporation of precursors into macromolecules. At that time it was difficult to determine if the enterotoxin-induced inhibition of incorporation was due to an effect(s) of enterotoxin on energy metabolism, precursor transport, membrane permeability or macromolecular synthesis. The high rates of leakage of nucleotides from enterotoxin-treated growing cells strongly suggests that altered membrane permeability alone could explain the apparent cessation of incorporation. If membrane permeability was altered such that most or all precursors and ions leaked from enterotoxin-treated cells, macromolecular synthesis would soon cease. We propose that the primary action of the enterotoxin is to interact with the plasma membrane and rapidly induce permeability alterations. As a result, vital processes, such as macromolecular synthesis, soon shut down and this leads to cell death.

Lastly, *C. perfringens* has many properties which distinguish it from other enterotoxins [25]. *Escherichia coli* enterotoxin, for example, does not induce aminoisobutyric acid leakage [10]. Because of its uniqueness among enterotoxins and apparent involvement with membranes, elucidation of the mode of action of *C. perfringens* enterotoxin could lead to a better understanding of membrane structure-function relationships, particularly with regard to the intestinal brush order, which is thought to be the site of action of *C. perfringens* intestinal disease [25].

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