Permeabilization of Rat Hepatocytes with *Staphylococcus aureus* α-Toxin

BRUCE F. McEWEN and WILLIAM J. ARION

Division of Nutritional Sciences and the Section of Biochemistry, Molecular and Cell Biology of the Division of Biological Sciences, Cornell University, Ithaca, New York 14853. Dr. McEwen's present address is Division of Laboratories and Research, New York State Department of Health, Albany, New York, 12201.

ABSTRACT Pathogenic staphylococci secrete a number of exotoxins, including α-toxin. α-Toxin induces lysis of erythrocytes and liposomes when its 3S protein monomers associate with the lipid bilayer and form a hexomeric transmembrane channel 3 nm in diameter. We have used α-toxin to render rat hepatocytes 93–100% permeable to trypan blue with a lactate dehydrogenase leakage ≤22%. Treatment conditions included incubation for 5–10 min at 37°C and pH 7.0 with an α-toxin concentration of 4–35 human hemolytic U/ml and a cell concentration of 13–21 mg dry wt/ml. Scanning electron microscopy revealed signs of swelling in the treated hepatocytes, but there were no large lesions or gross damage to the cell surface. Transmission electron microscopy indicated that the nucleus, mitochondria, and cytoplasm were similar in control and treated cells and both had large regions of well-defined lamellar rough endoplasmic reticulum. Comparisons of the mannose-6-phosphatase and glucose-6-phosphatase activities demonstrated that 5–10 U/ml α-toxin rendered cells freely permeable to glucose-6-phosphate, while substantially preserving the selective permeability of the membranes of the endoplasmic reticulum and the functionality of the glucose-6-phosphatase system. Thus, α-toxin appears to have significant potential as a means to induce selective permeability to small ions. It should make possible the study of a variety of cellular functions in situ.

The study of the relationship of enzymic activities to internal cell structure requires the development of assay systems that can determine these activities in situ. For most reactions, the development of such assays requires circumventing the permeability barrier presented by the plasma membrane. The use of toluene (1, 2) or detergents to render the plasma membrane permeable to ionic substrates is unsatisfactory since such agents induce permeability changes in intracellular membranes. Toluene treatment also results in large morphological changes in the internal cell structure (2). The ideal reagent would be one that selectively reacts with the plasma membrane, does not induce any alterations of cellular morphology, and does not cause leakage of cytosolic enzymes.

Filipin (3–7), digitonin (8–12), and saponin (13–15), all of which complex with membrane-bound cholesterol, have been used to create permeability in cells to a variety of substances. Since the plasma membrane has a higher cholesterol content than most internal membranes (e.g., mitochondria and the endoplasmic reticulum [ER][1]), it has been postulated that these agents would react preferentially with the plasma membrane (6, 7, 11, 13). In spite of this selective interaction, the use of filipin or digitonin can cause extensive changes in cell morphology (12, 16, 17).[2] The rough ER is especially vulnerable, and other studies show that these agents alter the kinetics of ER enzymes (Lange, A. J., B. F. McEwen, and W. J. Arion, unpublished observations; and references 18–20), which suggests the agents interact with the ER membrane or ER proteins. Because of the structural similarity of saponin to digitonin (21), saponin interaction with the ER membrane is also expected. Thus, these agents are unsatisfactory for use in the in situ characterizations of at least some enzymic activities in

[1] *Abbreviations used in this paper:* ER, endoplasmic reticulum; LDH, lactate dehydrogenase.

[2] McEwen, B. F., C. T. Handelman, J. N. Telford, and W. J. Arion. Manuscript submitted for publication.

THE JOURNAL OF CELL BIOLOGY · VOLUME 100 JUNE 1985 1922–1929

the hepatocyte ER (namely glucose-6-phosphatase and UDP-glucuronyltransferase).

α-Haemolysin, or α-toxin, is an exotoxin secreted by pathogenic staphylococci that causes lysis of erythrocytes (22–24). α-Toxin forms characteristic ring structures in the membranes with which it interacts (25, 26). Füssle et al. (27) demonstrated that the rings, 2.5–3.0 nm in diameter, are hexomers of a 34-kD protein that is the α-toxin monomer. The hexomers induce resealed ghosts to leak inulin (5 kD) but not myoglobin (17 kD).Thelestam and Möllby (28) demonstrated that in contrast to more detergent-like cytolytic agents, α-toxin (and similar pore-forming agents) causes the leakage of small ions (e.g., α-aminoisobutyric acid, molecular weight 103) from human diploid fibroblasts without causing the leakage of polynucleotides (<10 kD) or RNA (>200 kD). Cassidy et al. (29) exploited this characteristic of α-toxin to study the effect of Ca^{+2}, Sr^{+2}, and ATP on tension and relaxation of rabbit ileum smooth muscle.

Since Bernheimer et al. (26) observed that α-toxin reacted with rat hepatocyte plasma membranes to form the characteristic rings, we reasoned that it could be used to render hepatocytes permeable to ionic substrates while retaining cytosolic enzymes. Because it is a pore-forming agent, it seemed likely that the action of α-toxin would be more specific and more easily controlled than that of filipin or digitonin which form large irregular lesions in the hepatocyte plasmalemma (5, 12, 17, 21).[2] This report documents the interactions of α-toxin with rat hepatocytes. A preliminary report describing portions of this study has been presented (30).

MATERIALS AND METHODS

Materials: α-Toxin was a generous gift from Behringwerke (Marburg, West Germany). Filipin was a gift from the Upjohn Co. (Kalamazoo, MI). Collagenase type Cls II was obtained from Worthington Biochemical Corp. (Freehold, NJ). Fixatives and other electron microscopy supplies were obtained from either Electron Microscopy Sciences (Ft. Washington, PA) or Ted Pella, Inc. (Irvine, CA). ^{32}P-labeled hexose-6-phosphates were prepared as described previously (31). Glutathione, saponin, ATP, NADH, NADPH, trypan blue, and lactate dehydrogenase (LDH) assay reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Cacodylic acid, also from Sigma Chemical Co., was recrystallized from 95% ethanol (32). Other chemicals were the purest commercial grade available.

The crude, lyophilized α-toxin preparation was >90% protein, as determined by the biuret procedure (33). The preparation was assayed for hemolytic activity with human erythrocytes, as described by Bernheimer and Schwartz (34) and was found to have 4.7 U/mg of a dry (lyophilized) α-toxin preparation. From Wadström's data that compares the hemolytic potency of α-toxin with human and rabbit erythrocytes (35), this is equivalent to ~400–500 U of rabbit hemolytic activity per milligram of protein, or roughly equivalent to the stage three α-toxin described by Bernheimer and Schwartz (34). Stock α-toxin solutions (94 U/ml) were prepared by dissolving 20 mg of toxin in 1 ml of suspension buffer.

Preparation of Hepatocytes: The animals used in these studies were either Sprague-Dawley or Long-Evans male rats (Blue Spruce Farms, Inc., Altamont, NY) weighing between 200 and 350 g. Animals were housed individually in wire cages under a 12:12 h light/dark cycle and fed *ad libitum* Agway Pro Lab (14% protein, 6% fat, 75% carbohydrate, and 4.5% fiber) (Agway Inc., Syracuse, NY). Hepatocytes were prepared by the method of Berry (36), as modified by Cornell et al. (37). The perfusion media contained 0.03–0.04% (wt/vol) collagenase without hyaluronidase in Ca^{+2}- and Mg^{+2}-free Krebs-Henseleit buffer. The perfusion was continued for 25–30 min at 37°C. The hepatocytes were liberated from the digested liver by gentle teasing, filtered through a 250-μm nylon mesh, and collected by centrifugation (1.5 min at 33 *g*). The cells were washed three times with complete Krebs-Henseleit buffer (containing Ca^{+2} and Mg^{+2}) and resuspended in media described below. Cell viability, as judged by trypan blue exclusion, was typically between 90 and 95%.

Preparation of Permeabilized Cells: For treatment with α-toxin, the washed hepatocytes were suspended to ~140 mg/ml wet wt in a buffer, pH 7.0, containing 20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), 250 mM mannitol, 3 mM $MgCl_2$, 3 mM ATP, and 5 mM reduced glutathione. Between 0.04 and 0.20 ml of the α-toxin stock solution was added to 0.05 or 0.25 ml of hepatocyte suspension, and suspension buffer was added to give a final incubation volume of 0.40 ml. The hepatocyte suspension was then flushed with 95:5 O_2/CO_2 and placed in a shaking water bath at 37°C for the indicated times.

The procedures for treatments with filipin described by Jorgenson and Nordlie (6) and Gankema et al. (7) were followed as closely as possible.

Trypan Uptake and Leakage of Lactate Dehydrogenase: Uptake of trypan blue was measured as the percentage of cells stained with dye as scored under the optical microscope. LDH activity was assayed in cell-free supernatants according to Sigma Technical Bulletin No. 340UV (Sigma Chemical Co.) with the following modifications: temperature correction was ignored; the assay volume was scaled down to 1.0 ml; and NADH levels were adjusted to give a starting optical density of ~1.0. To determine total hepatocyte LDH activity (i.e., 100% leakage), hepatocytes were exposed at 37°C either for 20 min to 500 μM filipin or for 10 min to 500 μg/ml saponin. Cell-free supernatants were obtained by centrifuging 0.1 ml of cell suspension for 15 s at 9,000 *g* in a Beckman Microfuge B (Beckman Instruments, Inc., Fullerton, CA), and an 0.05-ml aliquot of the supernatant was diluted to 0.5 ml with 0.1 M potassium phosphate, pH 7.5.

Hexose-6-phosphatase Assays: To assay phosphohydrolase activities with 2 mM mannose-6-phosphate or 2 mM glucose-6-phosphate hepatocytes were exposed to α-toxin for 5 min at 37°C in a buffer system (denoted medium C) that contained 5 mM KP_i (P_i, inorganic phosphate), 3 mM ATP, 0.5 mM ADP, 5 mM MgC_2, 0.5 mM NAD, 0.125 mM NADP, 5 mM reduced glutathione, 4 mM lactate, 5 mM alanine, 10 mM glucose, 140 mM mannitol, 0.48 mM $CaCl_2$, 1.0 mM EGTA, 51.3 mM KCl, and 2% (wt/vol) bovine serum albumin (defatted) at a final pH of 7.0. Immediately after toxin treatment, 20 μl of cell suspension was added to 80 μl of medium C that had been supplemented to 2 mM ^{32}P-labeled hexose-6-phosphate and equilibrated at 30°C. After 4 min at 30°C, the reaction was stopped by addition of 0.9 ml of 1.25 M perchloric acid that contained 0.25 mM KP_i and 0.2 mM KIO_3. A 0.5-ml aliquot of the protein-free supernatant was transferred to a 10 × 75-mm glass tube and mixed with 0.1 ml of 8% ammonium molybdate. The ^{32}P-phosphomolybdate was then quantitatively extracted into 0.9 ml of isobutanol/benzene, vol/vol (38).

Aliquots (0.5 ml) of the organic phase were mixed with 5 ml of Liquiscint (National Diagnostics, Inc., Sommerville, NJ), and radioactivity was measured in a Beckman Model LS2800 liquid scintillation counter (Beckman Instruments, Inc.). All samples were corrected for radioactive decay and for nonenzymatic hydrolysis of substrates by running "zero-time" controls, wherein the perchloric acid solution was added to the assay medium before the addition of cell suspensions.

Liver homogenates (10% wt/vol) were prepared in 0.25 M sucrose containing 5 mM HEPES, pH 7.4, as described previously (39). After diluting the homogenates with 10 vol of medium C, untreated homogenates were assayed as described for permeabilized cells. Liver preparations in which membrane permeability barriers were completely lacking (termed fully disrupted) were prepared as follows. Washed hepatocytes (~75 mg wet wt) were suspended in 1.0 ml of 50 mM Tris-cacodylate buffer, pH 7.0 (38). The cell suspension was sonicated at 23°C for 3 min at maximum power in a bath-type ultasonic cleaner (Model G112S1T, Laboratory Supplies, Inc., Hicksville, NY). A 50-μl aliquot of the sonic suspension was cooled to 0°C and diluted with 40 μl of ice-cold 50 mM Tris-cacodylate, pH 7.0, and supplemented to 0.2% sodium deoxycholate by addition of 10 μl of a 2% detergent solution. After 20 min on ice, 1.0 ml of medium C was added, and 20-μl aliquots were assayed for phosphatase activities as described above, except the assay period was increased to 10 min. Fully disrupted homogenates were prepared and assayed by processing 50 μl of homogenate exactly as described above for the sonicated cell suspension.

Electron Microscopy: Hepatocytes were fixed for electron microscopy with 2.5% glutaraldehyde (30–90 min at 4°C) and 1% osmium tetroxide (30 min at room temperature). The samples were then dehydrated with an ascending ethanol series. Usually a sample in the last absolute ethanol solution was divided so that one portion could be used for transmission electron microscopy and another portion could be used for scanning electron microscopy. For transmission electron microscopy, samples were embedded in Spurr media, and silver sections were cut with a diamond knife. Sections were stained with 2% alcoholic uranyl acetate (35% ethanol, 12.5% methanol), Reynolds lead citrate (40), and viewed at 80 kV with a Philips EM300 electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ). For scanning electron microscopy, the absolute ethanol was displaced by liquid carbon dioxide which served as the transitional fluid for critical point drying. Dried samples were mounted on aluminum stubs and coated with gold-palladium using a sputter coater. Samples were viewed with an AMR1000 scanning electron microscope (Amray, Inc., Bedford, MA).

RESULTS

Conditions for Treating Cells with α-Toxin

In a series of preliminary evaluations, we sought conditions whereby α-toxin might induce permeability in hepatocytes to trypan blue (molecular weight 1,000) but not to cytosolic enzymes such as LDH (molecular weight 140,000). Concentrations of α-toxin from 1 to 50 U/ml in suspensions of cells ranging between 5 and 21 mg dry wt/ml (18–73 mg wet wt/ml) were exposed for up to 30 min at 37°C. Permeability to trypan blue was not induced at room temperature (23°C) or below. These evaluations revealed that greatest permeability to trypan blue with minimal leakage of LDH could be achieved when moderate cell concentrations (10–21 mg dry wt/ml) were incubated at 37°C ≤10 min with relatively high concentrations of α-toxin (20–45 U/ml).

We recently evaluated two published procedures (6, 7) that use filipin to permeabilize rat hepatocytes (16, 17).[2] We have also used saponin, which has been reported to induce permeability in cells (13–15). Table I compares the effectiveness of α-toxin with other agents in inducing permeability to trypan blue in rat hepatocytes while maintaining low LDH leakage. Whereas the filipin treatment described by Gankema et al. (7) could be used to achieve this differential permeability, it was a difficult procedure to control, and lower LDH leakage was invariably accompanied by lower trypan blue uptake.[2] The data in Table I show that α-toxin is more effective than the other agents in inducing differential permeability.

Morphology of α-Toxin–treated Cells

Figs. 1 and 2 compare the internal morphology of hepatocytes before and after treatment with α-toxin. The micrographs for control hepatocytes shown in Fig. 1 agree well with accepted standards for the morphology of undamaged parenchymal cells (36, 41, 42). α-Toxin–treated hepatocytes (Fig. 2) show large areas of intact and unswollen granular ER. There is some loss of gray background in the cytosol compared with control cells, but this occurs to a much lesser extent than it does with filipin-treated cells.[2] Likewise, the mitochondrial swelling is less in these cells, and intact cristae are visible. The nuclear membrane is intact, although rearrangement of chromatin is seen in the nucleus proper. In general, the internal morphology of hepatocytes is reasonably well-preserved after exposure to α-toxin.

Scanning electron micrographs of control and toxin-treated cells are shown in Fig. 3, *A* and *B*. Many microvilli are still visible in both preparations. α-Toxin–treated cells appear swollen, but the large surface lesions observed after treatments with filipin (17)[2] or digitonin (12) are not seen.

Assays of Glucose-6-phosphatase Activity in α-Toxin–treated Cells

The hydrolytic component of the glucose-6-phosphatase system, which is localized on the lumenal aspect of the ER membrane and nuclear envelope, catalyzes the hydrolysis of both mannose-6-phosphate and glucose-6-phosphate (43–45). However, since these membranes are impermeable to mannose-6-phosphate (44–46), the expression of mannose-6-phosphatase activity occurs only if the permeability barrier defined by the ER membranes is damaged, such as by detergents, or otherwise modified such as could occur on reaction with α-toxin. Thus, assessments of the latency[3] of mannose-6-phosphatase activity provide a means to quantify the extent of disruption or permeabilization of ER membranes (44, 45).

Part *A* of Table II summarizes the effects of α-toxin concentration on the uptake of trypan blue and phosphohydrolase activities with mannose-6-phosphate (M6Pase) and glucose-6-phosphate (G6Pase). Part *B* gives enzyme activities in total liver homogenates prepared from littermates and assayed without prior exposure to α-toxin. In control cells incubated in the absence of α-toxin, <20% of total cellular glucose-6-phosphatase activity was expressed. This agrees with the percentage of cells stained with trypan blue. The glucose-6-phosphatase activity increased with increasing α-toxin until maximal expression was achieved at 4.7 U/ml. In contrast, complete staining with trypan blue was not obtained even at the highest concentration of toxin used. The highest rate of glucose-6-phosphate hydrolysis obtained in permeabilized cells was in good agreement with the value obtained with untreated homogenates.

Except at the highest concentration of α-toxin, the mannose-6-phosphatase activity in toxin-treated cells or untreated homogenates was only ~10% of the activity with glucose-6-phosphate. This should be compared with the value of near 70% observed with fully disrupted cell or homogenate preparations. Moreover, over this range of toxin concentration, the mannose-6-phosphatase activity was <10% of the maximal potential for mannose-6-phosphate hydrolysis expressed in cells or homogenates after disruption with sodium deoxycholate.

Finally, the internal morphology of cell preparations that

[3] Latency is defined as the percentage of activity in fully detergent-disrupted preparations that is not expressed in preparations not exposed to detergents. Latency is calculated as 100 (detergent-treated activity − untreated activity)/detergent-treated activity.

TABLE I

Trypan Blue Uptake and LDH Leakage in Rat Hepatocytes Treated with Filipin, Saponin, or α-Toxin

Agent	Agent concentration	Cell concentration	Treatment time	Trypan blue uptake	LDH leakage
		mg dry wt/ml	*min*	*%*	*%*
None	0	21	0	9 ± 2	8 ± 2
None	0	21	30	16 ± 4	19 ± 2
Filipin I*	100 μM	1	10	100	90–100
Filipin II‡	50 μM	20	1	75–90	25–40
Saponin	80 μ/ml	13–16	5	90–100	60–90
α-Toxin	35 U/ml	13	5	93	12
α-Toxin	35 U/ml	13	10	100	22

* From the procedure of Jorgenson and Nordlie (6).
‡ From the procedure of Gankema et al. (7).

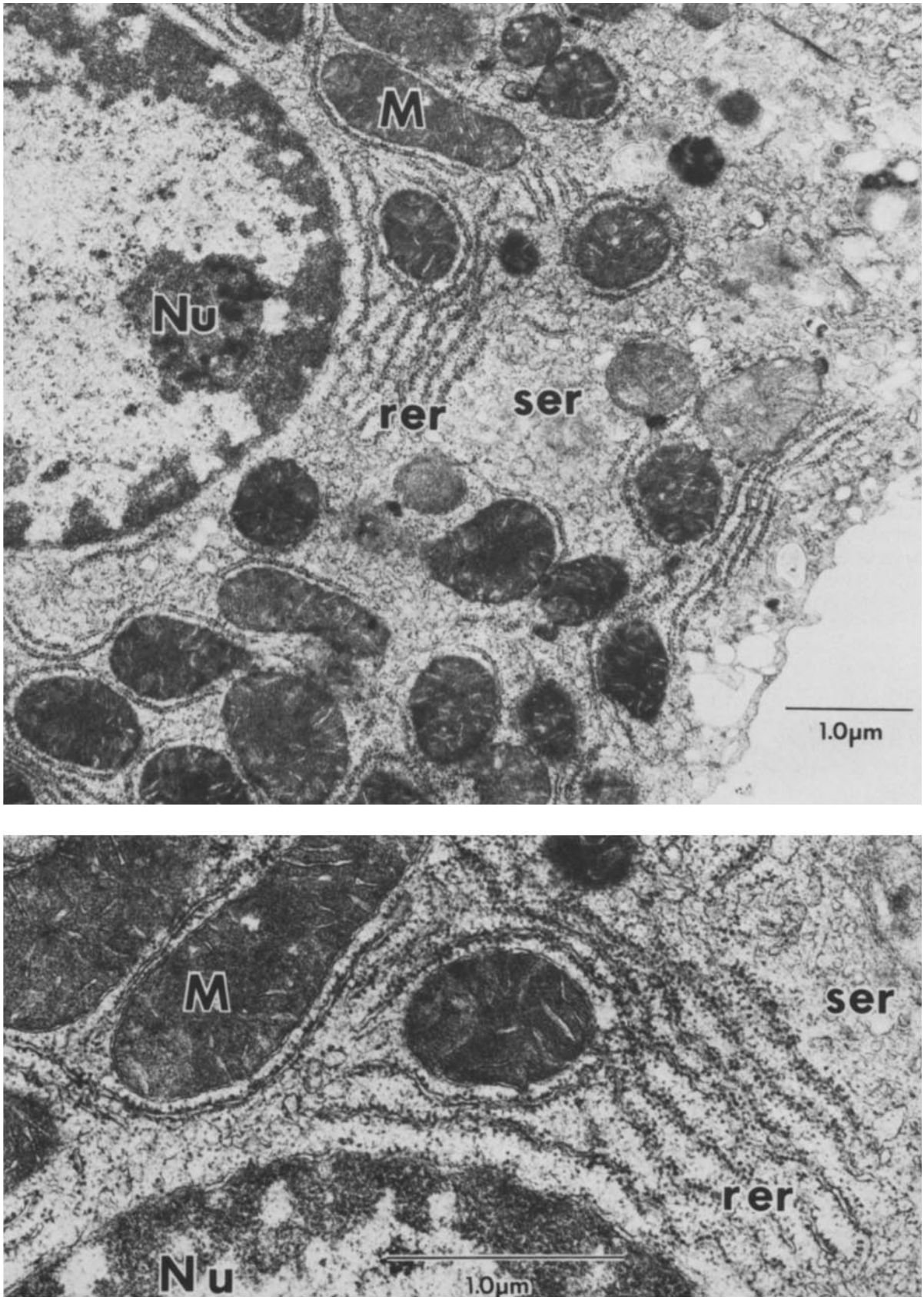

FIGURE 1 Control hepatocytes in Krebs-Henseleit butter. Freshly isolated hepatocytes were prepared for electron microscopy as described in Materials and Methods. *M*, mitochondria; *Nu*, nuclei; *rer*, rough endoplasmic reticulum; *ser*, smooth endoplasmic reticulum. Bars, 1.0 μM.

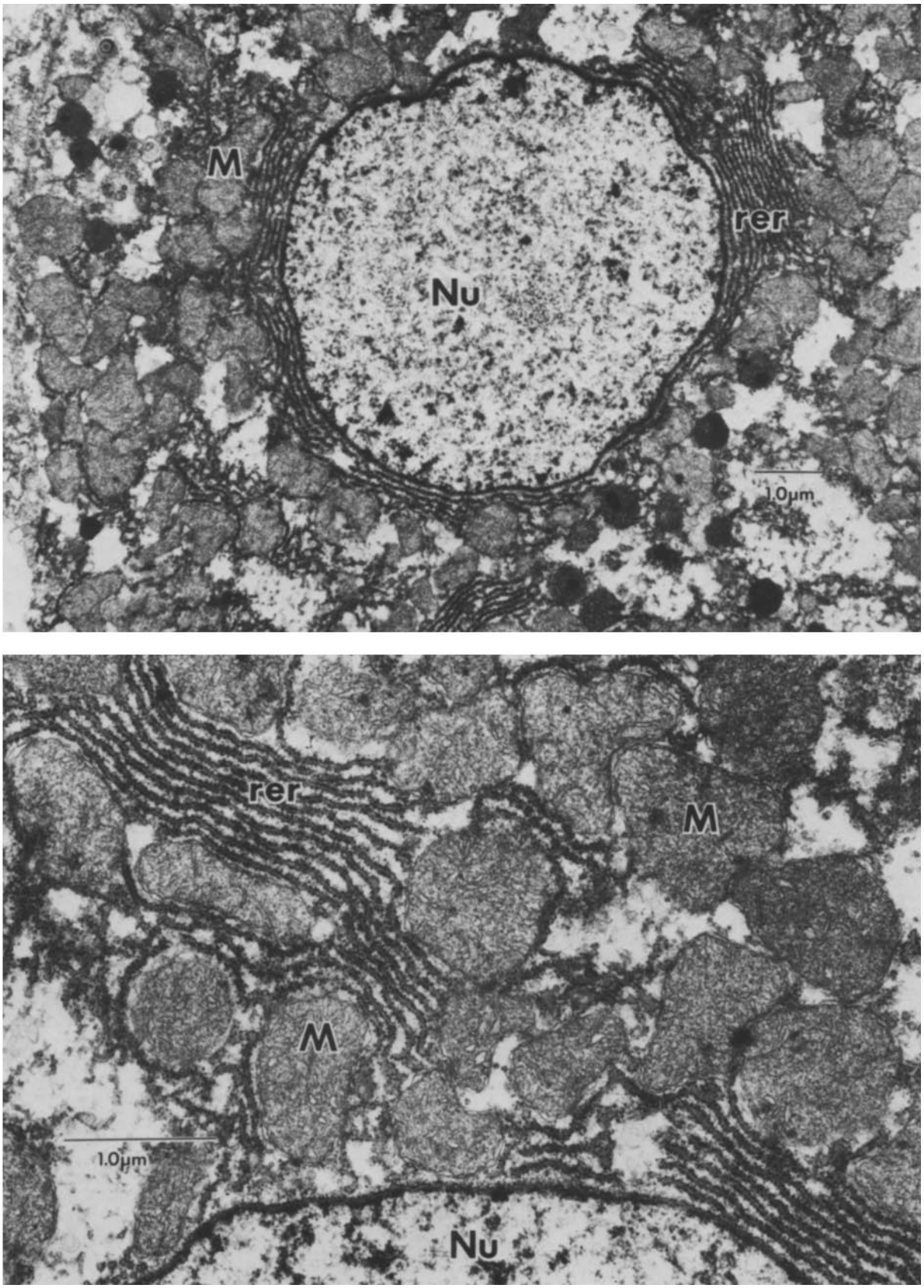

FIGURE 2 Hepatocytes treated with α-toxin. The concentrations of α-toxin and cells during treatment were 35 U/ml and 16 mg dry wt/ml, respectively. After a 10-min incubation at 37°C, the hepatocytes were fixed for electron microscopy. *M*, mitochondria; *Nu*, nuclei; *rer*, rough endoplasmic reticulum. Bar, 1.0 μM.

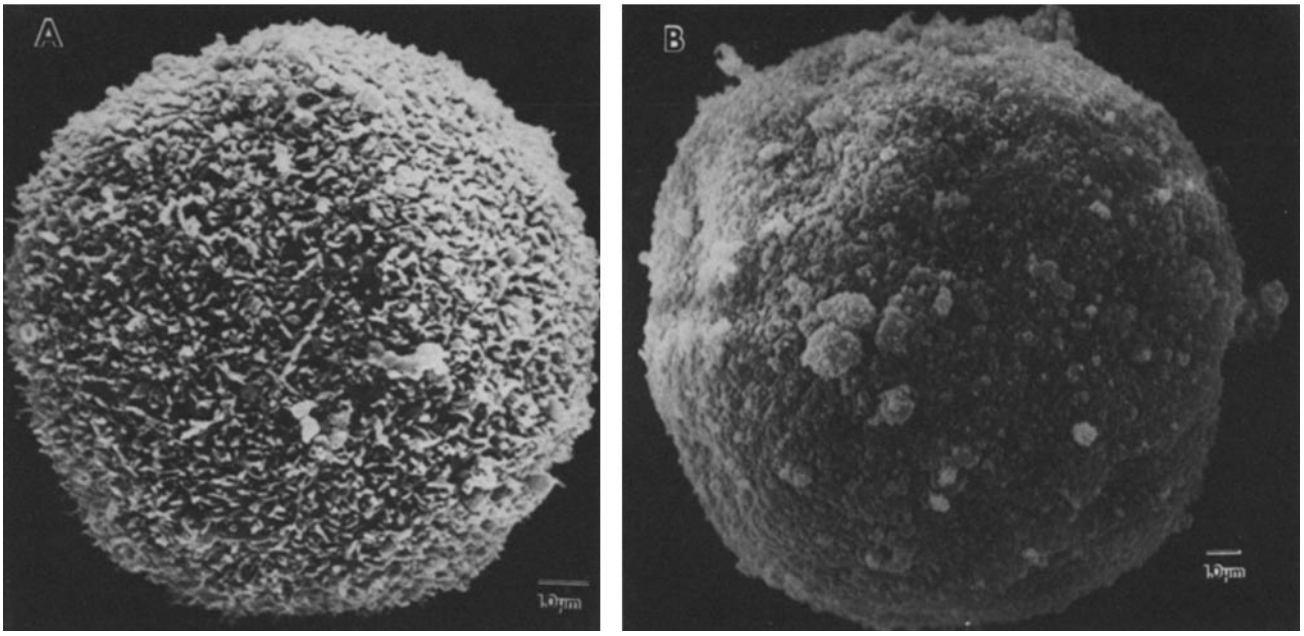

FIGURE 3 Scanning electron micrographs. (*A*) Control hepatocytes. (*B*) Hepatocytes treated with α-toxin as described in Fig. 2. Bars, 1.0 μM.

TABLE II

Trypan Blue Uptake and Hexose-6-phosphatase Activities with Glucose-6-phosphate (G6Pase) and Mannose-6-phosphate (M6Pase) in Hepatocytes Exposed to Various Concentrations of α-Toxin

	α-Toxin	M6Pase activity	G6Pase activity	M6Pase / G6Pase	Trypan blue uptake
	U/ml	*μmol/min/g wet wt*			*%*
A. Hepatocytes	0	0.11	0.98	0.11	18
	0.047	0.12	1.2	0.11	21
	0.14	0.16	1.4	0.12	18
	0.35	0.18	1.4	0.13	20
	0.94	0.32	2.6	0.12	27
	1.9	0.43	4.6	0.09	26
	2.4	0.37	4.6	0.08	21
	4.7	0.40	5.0	0.08	29
	9.4	0.39	4.9	0.08	45
	18.8	0.50	5.1	0.10	70
	37.6	0.90	5.2	0.17	94
	0				
	Fully disrupted*	5.4	7.9	0.72	—
B. Homogenates	Untreated	0.52	5.3	0.10	—
	Fully disrupted*	4.7	6.8	0.69	—

Mean values for triplicate assays from two experiments are presented. In part *A*, hepatocytes from fed rats (22 mg dry wt/ml) were exposed for 5 min to the indicated concentration of α-toxin and assayed for phosphohydrolase activities, as described in Materials and Methods. Phosphatase activities measured in total liver homogenates from fed littermates of the rats used in part *A* are presented in part *B*.

* The designation "Fully disrupted" indicates that liver preparations were treated with 0.2% sodium deoxycholate before the conduct of enzymic assays (see Materials and Methods).

had been exposed for 5 min at 37°C to α-toxin (2.4–9.4 U/ml) and then incubated for 3–5 min at 30°C with either 2 mM glucose-6-phosphate or 2 mM mannose-6-phosphate was indistinguishable from that shown in Fig. 2. These findings indicate that under conditions in which α-toxin induced maximal permeability of the plasma membrane to glucose-6-phosphate, both the native permeability of the ER membranes and the functionality of the glucose-6-phosphatase system were substantially preserved.

DISCUSSION

This report presents evidence that α-toxin can be used to render rat hepatocytes permeable to trypan blue and glucose-6-phosphate under conditions which preserve the selective permeability of the ER membranes and cause only low leakage of LDH and minimal disruption of internal morphology. To our knowledge, this is the first report demonstrating that α-toxin can be used to modify the permeability of the plasma membrane of hepatocytes, although this was suggested by the study of Bernheimer et al. (26). Since α-toxin can induce permeability in liposomes (26, 27), the agent does not require a protein receptor to interact with lipid bilayers. However, the hemolytic potency of α-toxin varies widely with erythrocytes from different species (34, 35), and Bernheimer et al. (22, 26) found several bacterial membranes that would not react to form characteristic ring structures with α-toxin. These

observations suggest that the interaction of the toxin with membranes is influenced by the resident lipids and proteins. Accordingly, the appropriate titer for α-toxin must be empirically established for each cell type.

The assays of glucose-6-phosphatase and mannose-6-phosphatase (Table II) demonstrated that α-toxin can induce high permeability of the plasmalemma to glucose-6-phosphate without significant changes in the selective permeability of the ER membranes. In control experiments (data not shown), we incubated homogenates (20 mg wet liver/ml) for 4 min with either 9.4 or 23.5 U/ml of toxin. At the lower concentration of toxin, rates of mannose-6-phosphate or glucose-6-phosphate hydrolysis were not significantly altered from control homogenates incubated without added toxin, but at the higher concentration of toxin, mannose-6-phosphatase activity was substantially elevated, approaching 50% of the activity observed in fully disrupted homogenates. These data indicate that α-toxin can induce permeability to mannose-6-phosphate when given direct access to the ER membranes. By comparison, only modest activations of mannose-6-phosphatase were observed when cells were treated at a higher concentration of toxin (see Table II). These data lead us to conclude that for the most part, α-toxin does not penetrate into the interior of the cell. The modest activation of mannose-6-phosphatase activity noted in cells exposed to higher concentrations of toxin most likely reflects permeabilization of the ER membranes localized in the small subfraction of cells that have large surface lesions, e.g., those cells from which LDH leakage occurred.

The good agreement between the glucose-6-phosphatase activity in homogenates and in α-toxin–treated cells indicates that (*a*) permeabilization can be achieved without loss of functionality of the glucose-6-phosphatase system and (*b*) potential kinetic barriers presented by the α-toxin pores or cytoplasmic diffusion gradients do not impose significant rate restrictions on the glucose-6-phosphatase system in situ. These encouraging findings have prompted us to undertake a comprehensive characterization of the glucose-6-phosphatase system of these cells.

Maximal glucose-6-phosphatase activity with 2 mM substrate was obtained with <5 U/ml of α-toxin. Under these conditions, significant staining with trypan blue was observed in only 30% of the cells. Since the α-toxin pore should be large enough to accommodate trypan blue (26–28), the reason for the apparent differential permeability to glucose-6-phosphate and trypan blue is not obvious. It may simply reflect the difference in the relative ability (i.e., sensitivity) of the radiochemical and visual methods to detect penetration into the cells. However, others have found that the amount of α-toxin required for marker release from erythrocyte ghosts (27) or human fibroblasts (28) depends upon the size of the marker. In any case, since most intracellular metabolites are smaller than trypan blue (molecular weight 1,000), the conditions described in Table I probably represent the extreme in what is necessary to achieve high rates of metabolite permeation into rat liver hepatocytes. Again, we note the necessity of titrating the concentration of α-toxin required for the system under study.

Whereas differential permeabilization was easily achieved with α-toxin, some increase in LDH leakage was consistently noted with increasing time of exposure (Table I). Leakage of LDH from permeabilized cells during longer incubations with α-toxin could be related to the osmotic swelling evidenced in Fig. 3*B*. In fact, osmotic swelling is the mechanism by which α-toxin and complement lyse erythrocytes. Lysis by the latter has been prevented by the addition of high concentrations of agents that will not pass through the complement pore (e.g., bovine serum albumin) (47, 48). Alternatively, increased LDH leakage could be secondary to the loss from the cell of low molecular weight cofactors which are necessary for the maintenance of cell integrity. The inclusion of Mg^{++}, ATP, and glutathione in our incubation medium stemmed from the finding of Gankema et al. (7) that these factors reduced LDH leakage from filipin-treated cells. Thus, it may be possible to limit the time-dependent breakdown of the cells by further modification of the incubation medium. Indeed, the question of what constitutes an appropriate intracellular medium remains to be definitively addressed.

We are grateful to Drs. Zwisler and Hungerer of the Behringwerke, Marburg, West Germany, for providing us with the preparation of lyophilized α-toxin used in these studies. We thank Alex J. Lange for his excellent technical assistance.

This work was supported by National Institutes of Health Research Grant AM-19625 and National Institutes of Health Postdoctoral Fellowship AM-07174.

Received for publication 5 September 1984, and in revised form 28 February 1985.

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