

CHARACTERIZATION OF A MEMBRANE PORE-FORMING PROTEIN FROM *ENTAMOEBEA HISTOLYTICA**

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Entamoeba histolytica, an enteric human parasite, produces extensive tissue destruction in the course of the infection. Prior studies from this laboratory have discussed the isolation of the plasma membrane of this parasite and characterized certain of its intrinsic polypeptides and externally disposed antigens (1, 2). More recent interests have focused on the role of the plasma membrane in the cytotoxicity of amoebae. Here, close apposition between amoeba and target cell membranes are a prerequisite for the extensive cytolysis seen on a wide spectrum of cell types (3-8).

In this study we deal with one component of the membrane of *E. histolytica* that may be involved in the cytotoxic mechanism. We have partially purified a polypeptide that inserts into membrane bilayers and opens aqueous pores. The conductance changes that this pore-forming material (PFM)¹ mediates can be resolved into individual ion channels.

Materials and Methods

Cells. The *E. histolytica* strains HK9:NIH and HM1:NIH, originally obtained from Dr. L. S. Diamond, were generously supplied by Dr. S. B. Aley and Dr. B. Arrick, The Rockefeller University. Maintenance in Diamond's TYI-S-33 medium was as described (1).

The J774 macrophage cell line was grown in Dulbecco's Modified Eagle's medium supplemented with 5% heat-inactivated fetal calf serum (FCS) (9). BALB/c mouse spleen lymphocytes enriched in T cells were prepared by nylon wool filtration (10).

Isolation of PFM from Amoebae. Amoebae were harvested at the end of log phase by chilling on ice for 5 min, which detached the cells from the glass surface. Cells were spun at 200 *g* (5 min, 4°C), washed twice with a phosphate-buffered saline buffer and pooled. Cells were then lysed by two cycles of freezing and thawing. To sediment nuclei, the cell suspension was centrifuged at 1,500 *g* (10 min, 4°C). The supernatant was immediately spun at 100,000 *g* (90 min, 4°C). The final supernatant (or the membrane pellet solubilized in 60 mM octylglucoside [Sigma Chemical Co., St. Louis, MO] as indicated in the text) was then used for isolation of PFM or for assay in the planar bilayer or [³H]tetraphenyl phosphonium⁺ ([³H]TPP⁺) equilibrium systems.

Aliquots (0.2 ml) of the final supernatant (or of detergent-solubilized membrane pellet) at

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¹ *Abbreviations used in this paper:* BSA, bovine serum albumin; Con A, concanavalin A; ΔΨ, membrane potential; FCS, fetal calf serum; *g*, conductance; *I-V*, current-voltage; LPS, lipopolysaccharide; *M_r*, molecular weight; PFM, pore-forming material; [³H]TPP⁺, [³H]tetraphenylphosphonium⁺; TYI-S-33, trypticase-yeast-iron medium.

protein concentrations of 5–10 mg/ml were applied to a 7.5- × 600-mm TSK-G3000 SW column (LKB, Produkter AB, Sweden) and eluted with 100 mM sodium phosphate buffer, pH 6.5, at a flow rate of 0.12 ml/min. Fractions of 0.3 ml were collected. Protein determination was done by a Coomassie blue binding assay (11).

Buffers. The following buffers were used for [^3H]TPP $^+$ uptake and planar bilayer experiments. Buffer A (low- K^+ buffer): 118 mM NaCl, 5 mM KCl, 1.3 mM CaCl_2 , 0.8 mM MgSO_4 , 5.5 mM glucose, 20 mM Hepes, 9 mM Na_2CO_3 , pH 7.4. Buffer B (high- K^+ buffer): buffer A, in which 118 mM KCl replaced NaCl. Buffer C: 0.05 M KCl (or NaCl), 0.165 M sucrose, 10 mM Hepes, adjusted to pH 7.4 with Tris base. Buffer D: 0.25 M sucrose, 10 mM Hepes-Tris, pH 7.4. Buffer E: 0.1 M KCl, 3 mM NaN_3 , 10 mM Tris-Cl, pH 7.4. Buffer F: 0.15 M NaCl, 4 mM MgCl_2 , 3 mM CaCl_2 , 3 mM NaN_3 , 10 mM Tris-Cl, pH 7.4.

[^3H]TPP $^+$ Uptake by Cells. Equilibration of J774 macrophage cells and mouse spleen lymphocytes with [^3H]TPP $^+$ as a probe for membrane potential was done essentially as described (12, 13). Cells (4×10^7 cells/ml, 0.1 ml) were diluted into 0.9 ml of buffer A or B containing 20 μM [^3H]TPP $^+$ (0.125 Ci/mmol, as bromide salt; a generous gift from Dr. H. R. Kaback, Roche Institute of Molecular Biology) and pre-equilibrated for 25 min before addition of amoeba extracts. Cells were agitated gently at 37°C throughout the experiment. At intervals after addition of amoeba extracts, 0.1-ml aliquots of the reaction mixture were filtered on a glass microfiber filter (934-AH; Whatman Laboratories, Inc., Clifton, NJ). The filter was immediately washed with 5 ml of Buffer A or B and transferred to vials containing 8 ml of Hydrofluor (National Diagnostics, Somerville, NJ) for assay of radioactivity by liquid scintillation spectrometry.

Determination of Cell Volumes. The cell size was determined with [^3H]H $_2\text{O}$ and [^{14}C]sorbitol as described (12) or by a Coulter Counter (with a Coulter Channelyzer, model H4; Coulter Electronics Inc., Hialeah, FL). Values determined by these two methods agreed within 10%. The volume per 10^6 cells was 1.5 μl for J774 cells and 0.2 μl for spleen lymphocytes.

Calculation of Membrane Potential ($\Delta\Psi$) from [^3H]TPP $^+$ Partition. The values of [^3H]TPP $^+$ accumulated in cells incubated in buffer B (high- K^+) were used as background, and were subtracted from [^3H]TPP $^+$ levels accumulated in cells incubated in buffer A (low- K^+). The corrected values were converted into concentration using the intracellular volumes mentioned above, and the concentration values were finally converted into millivolts of $\Delta\Psi$ by Nernst equation (12).

Preparation of Lipid Vesicles. Lipid vesicles were prepared by the detergent dialysis method (14). Egg phosphatidylcholine (type VI-E; Sigma Chemical Co.) and cholesterol (Sigma Chemical Co.) were mixed at the ratio of 6:1 (wt/wt) in chloroform/methanol (2:1), dried under a stream of N_2 , resuspended in diethyl ether, and dried again. To this mixture, buffer C containing 20 mM octylglucoside was added to make a final lipid concentration of 50 mg/ml. This solution was sonicated for 5 min in a water-bath sonicator and dialyzed against 1 liter of buffer C for 36 h, with three equal changes of the same buffer.

[^3H]TPP $^+$ Uptake by Lipid Vesicles. [^3H]TPP $^+$ associated with lipid vesicles was separated from free [^3H]TPP $^+$ by filtration through Millipore filters (0.2 μm , EGWP 02500; Millipore Corp., Bedford, MA) (15). The reaction was initiated by diluting 0.1 ml of lipid vesicles into 0.9 ml of buffer D, containing 50 μM [^3H]TPP $^+$. At intervals, 0.1-ml aliquots of reaction mixture were filtered; the filters were washed with 5 ml of ice-cold buffer D, and the radioactivity retained on the filter was determined. The amount of nonspecific binding of [^3H]TPP $^+$ to filters alone was subtracted from experimental values. PFM was added to buffer D before addition of the vesicle preparation. To assess the effect of valinomycin, vesicles made in buffer C with KCl were diluted 1:10 into buffer D containing NaCl, [^3H]TPP $^+$, and 10 $\mu\text{g}/\text{ml}$ of valinomycin (Sigma Chemical Co.).

Formation of Planar Bilayer Membranes and Electrical Measurements. Membranes were made by the technique of Montal and Mueller (16) spanning a 0.2-mm-diam hole in a Teflon partition (0.125 mm thick; Chemplast, NJ) separating two Teflon chambers (referred to as *cis* and *trans* compartments). The volume and the surface area of each chamber are about 6.5 ml and 6.5 cm^2 , respectively. The chambers and the partition were sonicated in chloroform/methanol (2:1, vol/vol) and 1 M HCl for at least 1 h to clean the chambers before each use. To make the membrane, the partition was first coated with squalene (1% in pentane; Sigma Chemical Co.).

The membrane was formed from a solution (10 mg/ml in pentane) of soybean phospholipid (lecithin, type II; Sigma Chemical Co.) from which neutral lipid had been removed (17). This solution (20 μ l) was layered over buffer E or F below the level of the hole contained in the Teflon partition. After evaporation of pentane (10 min later), the bilayer was formed by raising the level of the monolayers, one side at a time, above the level of the hole. Membrane formation was monitored electrically by an increase in capacitance.

Electrical measurements were made through a pair of saturated calomel electrodes (E-6A; Fisher Scientific Co., Pittsburgh, PA) which made contact with the buffer through KCl junctions. The voltage across the membrane was clamped through a direct current voltage supply. The current was fed into a current amplifier (model 427; Keithley Instruments Inc., Cleveland, OH) and the output was displayed on a dual-beam oscilloscope (type RM 502 A; Tektronix Inc., Beaverton, OR), a type 5113 storage oscilloscope (Tektronix Inc.) and/or registered on a recorder (model 2200 S; Gould Inc., Cleveland, OH). The conductance (g) of the membrane is defined as current divided by voltage and is given in Siemens (S, 1 S = 1 A/V); in the absence of any material derived from amoebae, $g = 5$ –10 pS. The potential of the *trans* side is defined as 0 (or virtual ground); thus, positive current flows from *cis* to *trans* side. Addition of PFM was always done through the *cis* side.

Enzymatic Digestions. Amoeba extracts were treated with 1 mg/ml trypsin (type III, Sigma Chemical Co.) for 1 h at 37°C, followed by 2 mg/ml of soybean trypsin inhibitor (1 h; type II-0, Sigma Chemical Co.). The extracts were then tested on cells or planar bilayers. To assay for the effect of pronase protease (from *Streptomyces griseus*, nuclease-free; Calbiochem-Behring Corp., La Jolla, CA), planar bilayers were prepared from PFM from amoebae added to the *cis* compartment. Pronase (1 mg/ml) was introduced either from the *cis* or *trans* side and the conductance through the bilayer was measured.

Secretion of PFM after Stimulation by Membrane-active Agents. Concanavalin A (Sigma Chemical Co.), lipopolysaccharide (LPS) from *Escherichia coli* (serotype 026:B6; Sigma Chemical Co.) and the calcium-ionophore A23187 (Calbiochem-Behring Corp.) were used to stimulate amoeba cell secretion of PFM. Drugs (0.1–5 μ g) were added to cultures containing 2×10^5 amoebae/ml. At intervals, culture supernatants (0.1 ml) after centrifugation (200 g , 5 min) were added to the *cis* compartment of planar bilayers and assayed for activity. The amount of activity retained in the cells was determined by washing the cells three times with TYI-S-33 medium, lysing the cells by freeze-thaw, and applying this material to a planar bilayer. Activity found in the supernatant was also given in terms of percentage of activity relative to that retained in the cell pellet.

Results

We have examined the effects of amoeba proteins on the ionic permeability of intact cells, lipid vesicles, and artificial planar bilayer membranes. Electrogenic ion flux induced by a PFM results in membrane potential changes that can be measured with the lipophilic cation [3 H]TPP $^+$ (12, 18). In addition, amoeba proteins were tested in planar bilayers—a system that provides evidence for the “opening” of single-channel events attributable to a PFM.

Effect of Amoeba Extracts on Membrane Potential ($\Delta\Psi$) of Cells. The resting $\Delta\Psi$, measured by the net accumulation of [3 H]TPP $^+$, was -14 ± 0.3 mV and -70 ± 1.1 mV for J774 macrophages and mouse spleen lymphocytes, respectively (Fig. 1). The addition of amoeba lysate (Materials and Methods) resulted in a drop of at least 9 and 40 mV in $\Delta\Psi$ for J774 cells and lymphocytes, respectively. A dramatic decrease in $\Delta\Psi$ was observable as early as 30 s after addition of amoeba extract, and the observed depolarization was of prolonged duration. After 50 min, the $\Delta\Psi$ of macrophages returned to baseline, whereas the $\Delta\Psi$ of lymphocytes did not normalize within 3 h. The depolarization-inducing activity was trypsin sensitive and heat labile (Fig. 1 a). Cells became depolarized when Na $^+$ was substituted by K $^+$ in the reaction buffer in which the cells were suspended. No additional depolarization was observed with

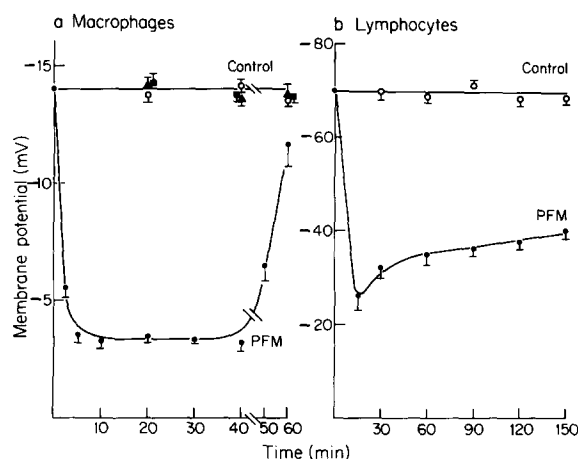


FIG. 1. Effect of amoeba extracts on membrane potential of cells. J774 macrophages (a) and mouse spleen lymphocytes (b) were equilibrated with $20 \mu\text{M}$ of $[^3\text{H}]\text{TPP}^+$ for 25 min before addition of amoeba extract at a final concentration of $50 \mu\text{g}$ protein/ml (\bullet); trypsin-treated extract (\blacktriangle); extract boiled for 5 min (\blacksquare); and control with no additions (\circ). Error bars represent the standard deviation of four experiments.

TABLE I
Amount of Activity Recovered in the Subcellular Fractions

Material	Conductance/ $5 \mu\text{g}$ protein
	<i>nS</i>
Freeze-thawed lysate	2.2 ± 0.45
Membrane pellet	2.4 ± 0.80
Supernate	0.8 ± 0.20
Column-purified PFM	620 ± 15
Dounced lysate	2.0 ± 0.52
Membrane pellet	2.5 ± 0.63
Supernate	0.3 ± 0.13

The activity was assayed in planar bilayer clamped at 30 mV 30 min after addition of $5 \mu\text{g}$ protein to 4–4.5 ml of buffer. Cells were lysed by freeze-thaw (two cycles) or douncing (10 strokes in a Potter-Elvehjem homogenizer), the post-nuclear supernate was then centrifuged at $100,000 g$ (90 min, 4°C), and the membrane pellet or supernate was collected for assay. Numbers refer to means of four experiments with standard deviation.

amoeba extract, which suggests that the depolarization is a passive process.

The most efficient method found to solubilize PFM from intact amoebae was to freeze-thaw the cells (2 cycles). The $100,000-g$ membrane pellet from the freeze-thaw lysate also retained PFM activity (Table I). Subsequent experiments were performed with the supernatant of a freeze-thaw lysate unless otherwise noted. The material was stable for up to 1 yr when stored at -70°C .

Effect of Amoeba Extracts on Ion Flow through Lipid Vesicles. The effect of amoeba extracts was next tested in an artificial lipid vesicle system. An ion gradient was introduced across the membrane by diluting vesicles equilibrated with 50 mM NaCl or KCl into equiosmotic solutions of sucrose containing $[^3\text{H}]\text{TPP}^+$. If net K^+ or Na^+ efflux occurred, the transient negative charge inside the vesicles would result in rapid uptake of $[^3\text{H}]\text{TPP}^+$. The addition of $20 \mu\text{g}/\text{ml}$ of amoeba protein resulted in a rapid

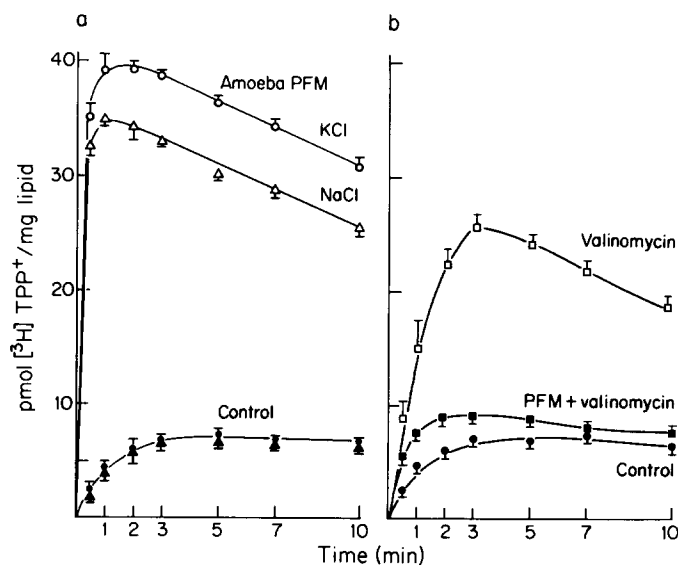


FIG. 2. (a) Treatment of lipid vesicles with amoeba extracts. Filtration experiments were done as described in Materials and Methods. KCl-loaded vesicles (\circ) and NaCl-vesicles (Δ) treated with 20 μg protein/ml of amoeba extract. Control experiments were done in the absence of amoeba material with KCl-loaded vesicles (\bullet) and NaCl vesicles (\blacktriangle). Points represent means of three experiments. (b) Vesicles (containing 50 mM KCl) were diluted into equimolar concentrations of NaCl, in the presence (\blacksquare) or absence (\square) of amoeba extract (50 $\mu\text{g}/\text{ml}$). 10 min later, the reaction was initiated with addition of 10 $\mu\text{g}/\text{ml}$ valinomycin and 50 μM $[^3\text{H}]\text{TPP}^+$ were added. The control (\bullet) was in the absence of valinomycin and amoeba extract. Points refer to means of four experiments.

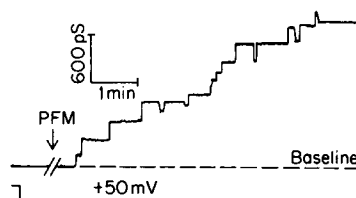


FIG. 3. Effect of amoeba extracts on planar bilayer membrane conductance. The artificial membrane was formed in symmetrical solutions of buffer E (0.1 M KCl). The voltage across the membrane was clamped at +50 mV (the *cis* side is positive), shown in the lower tracing. The electrical noise caused by the addition of amoeba extract (1 μg , to the *cis* side, indicated by the arrow) and subsequent stirring was deleted from the data.

accumulation of $[^3\text{H}]\text{TPP}^+$ that was five times greater than control values (Fig. 2a). Because greater efflux of K^+ or Na^+ than Cl^- occurred, the PFM in the amoeba lysate was cation selective.

We next compared the effect of PFM with the K^+ ionophore valinomycin. Whereas valinomycin could induce a rapid efflux of K^+ from KCl-loaded vesicles diluted into NaCl (not shown), as measured by uptake of $[^3\text{H}]\text{TPP}^+$, amoeba PFM discriminated poorly between K^+ and Na^+ and only small amounts of $[^3\text{H}]\text{TPP}^+$ were accumulated in the vesicles. Addition of PFM to KCl vesicles diluted into NaCl 10 min before the addition of valinomycin yielded only a small uptake of $[^3\text{H}]\text{TPP}^+$ in the vesicles (Fig. 2b). Thus, amoeba PFM acts as an ionophore, rapidly depleting the transmembrane K^+ - Na^+ ion gradient and abolishing the subsequent effect of valinomycin.

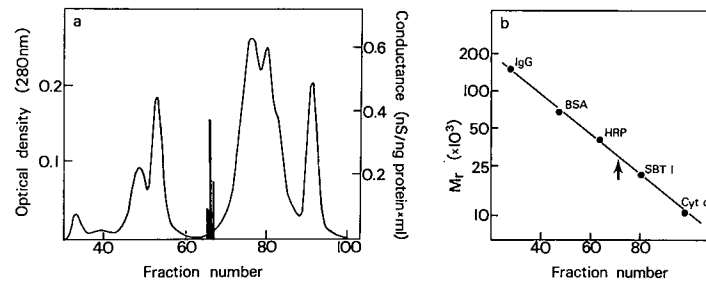


FIG. 4. (a) Gel filtration of amoeba lysate. Supernatant (0.2 ml, 5 mg/ml) from a 100,000-g freeze-thawed lysate was applied to a TSK-G3000 SW column as described. Aliquots (50 μ l) from each fraction were assayed in planar bilayer for pore-forming activity; scale in the right refers to membrane conductance 30 min after addition of each aliquot to bilayers made in 0.1 M KCl and clamped at constant 30 mV. (b) Plot of M_r standards vs. fraction number. PFM has a M_r of 30,000. Standards: rabbit gamma immunoglobulin (IgG), bovine serum albumin (BSA), horseradish peroxidase (HRP), soybean trypsin inhibitor (SBTI), and cytochrome *c* (CYT C).

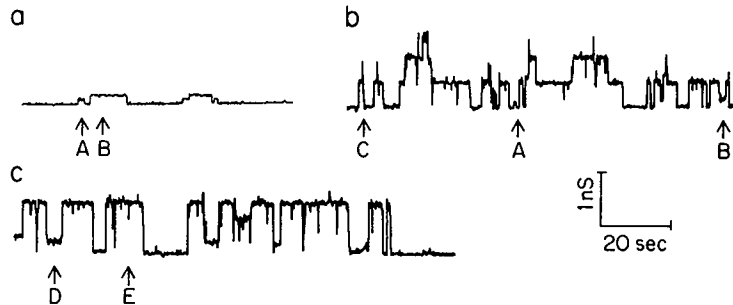


FIG. 5. Discrete conductance steps in planar bilayers induced by low amounts of PFM (10 pg). The membrane was made in buffer E (with KCl) and clamped at +30 mV; the same scale was used for comparison. (a) Unitary steps of 67 pS (A) and dimer of 135 pS (B) are indicated by arrows. (b) Three hexamers (C, 400 pS each), with less frequent smaller steps corresponding to monomers (A) and dimers (B). (c) Dodecamers (E, 800 pS). D points to a trimer.

Effect of Amoeba Extract on Planar Bilayer Membranes. To examine the molecular details of the permeability change, we used artificial planar bilayers and examined conductance changes with a voltage-clamp apparatus. Because of the higher impedance of planar bilayers, one can measure small conductance fluctuations with greater time resolution than with other techniques (16). The planar membranes showed a baseline conductance of 5–10 pS, which did not change in the presence of TYI-S-33 medium. However, addition of as little as 10 μ g of PFM increased the conductance of the bilayer by severalfold (Fig. 3). There was a progressive increase in conductance that exhibited discrete increments. Once inserted in the bilayer the conducting units were stable to rinsing and remained active in the absence of a membrane potential.

Partial Purification of PFM from Amoeba Extract. The PFM from *E. histolytica* was partially purified by gel filtration on a TSK-G3000 SW column (Fig. 4, Table I). The pore-forming activity was localized in a narrow band of M_r 30,000. When the amoeba lysate was stored at 4°C for 2 h, a PFM activity of $M_r > 200,000$ was found that could be disaggregated with 60 mM octylglucoside to the M_r 30,000 species. Membrane pellets solubilized with octylglucoside (see Methods) also showed activity of M_r 30,000. Column-purified PFM, when assayed with the planar bilayer system or [3 H]TPP $^+$ equilibration method, gave the same results as cell homogenates. Purification with

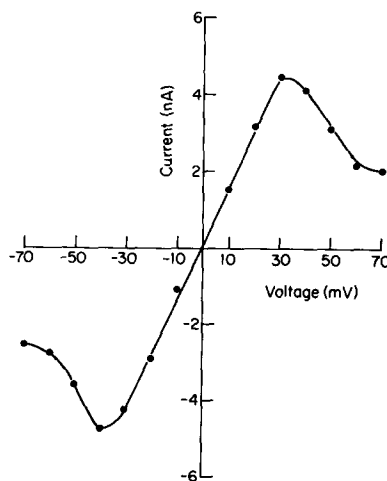


FIG. 6. The steady state current-voltage (I - V) plot of a membrane (buffer E, KCl) containing PFM. Voltage steps from -70 to $+70$ mV were applied across a membrane treated with $10 \mu\text{g}$ of partially purified PFM. The steady state values of current were measured after a 5-min exposure to each voltage step. Between every two voltage steps (10 mV intervals), voltage across the membrane was left at 0 for 1 min.

the one-step column elution method was on the order of $1,000 \times$ (Table I). The partially purified PFM was used for all subsequent electrophysiology experiments.

Single-Channel Fluctuations and Oligomerization of PFM in Planar Bilayers. Single-channel events were observed when low amounts of PFM (0.1 – 1 ng of protein) were used (Fig. 5). Unit conductance steps of 67 pS were rarely observed. More frequently, we saw dimers with a conductance of 135 pS, hexamers with a step of 400 pS, and dodecamers with a step of 800 pS, all of which turned on or off in synchrony. Less frequently, other intermediates (trimers, tetramers) were seen and may be due to preferential aggregation of individual conducting units. The height of the unit conductance steps was proportional to the ionic strength of the bathing buffer. In buffer F (physiological saline), the unit step was 90 pS.

Voltage and Concentration Dependence of PFM-induced Conductance. In planar bilayers containing large amounts of PFM, the application of a potential across the membrane led to immediate current flow, which behaved as an ohmic resistor. As the voltage increased, current increased proportionally. However, at higher voltages (>30 mV) the initial current then decreased with time to a lower steady state value within 5 min (Fig. 6). Thus, current flowing through the membrane at steady state increased linearly with the applied voltage, until a negative resistance region was reached.

We next studied whether the macroscopic voltage dependence of PFM could be explained by the behavior of single channels. Fig. 7 illustrates the behavior of the most frequent conductance step, the 400 -pS hexamer, as a function of two different voltages applied across the membrane. The current driven through each individual hexamer increased proportionally with higher voltage. However, the time in which the channel stayed open was voltage dependent, decreasing with higher voltages (Fig. 7). That is, increasing membrane voltage decreased the probability of finding the channel open, which resulted in the nonlinear I - V plot of Fig. 6. It is thus clear that voltage-dependent conductance could be satisfactorily explained by parameters that

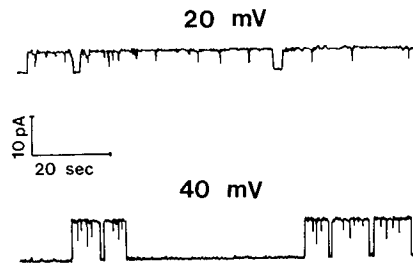


FIG. 7. Voltage-dependent conductance at the level of single channels. The membrane made in symmetrical 0.1 M KCl (buffer E) was treated with 5 μ g of partially purified PFM. The upward deflections are the openings of hexamers at two different voltages. Note that the current driven through each hexamer at 40 mV is twice that at 20 mV; however, the opening time is less at 40 mV.

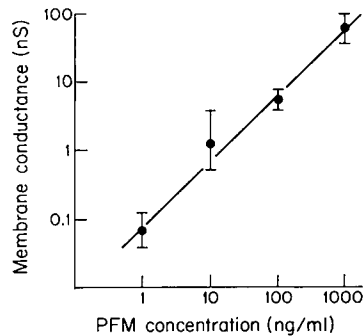


FIG. 8. Double-logarithmic plot of membrane conductance as a function of PFM concentration at a constant voltage (10 mV). Membranes (0.1 M KCl) were treated with different amounts of PFM and conductance measured 30 min after addition of material. Points represent means of four experiments.

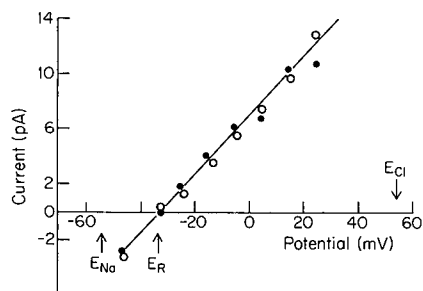


FIG. 9. Ion selectivity studies with PFM. A 10-fold gradient of NaCl was set up across the membrane (the *cis* side is more concentrated) before the addition of PFM (10 μ g). The reversal potential (E_R) at which current through PFM-treated membrane was abolished is shown. This value (-34 mV) was closer to the equilibrium potential for Na^+ (E_{Na}) than for Cl^- (E_{Cl}) (-54 and $+54$ mV, respectively). Data from two separate experiments are plotted.

describe single-channel kinetics.

Oligomerization of PFM was not required for the conducting state of PFM and monomers were functional. This was inferred from the double-logarithmic plot of conductance vs. PFM concentration, which gave a slope of 1 (Fig. 8). Such experiments were done with membranes clamped at 10 mV, that is, in the linear section of the I - V plot of Fig. 6.

Ion Selectivity and pH Dependence of PFM. To determine the anion or cation selectivity of PFM, the reversal potential to null current flow from a 10-fold-higher KCl or NaCl concentration (*cis* compartment more concentrated) was determined. The results (Fig. 9) show a reversal potential of -33 mV for KCl and -34 mV for NaCl. From the Nernst-Planck equation, a ratio of 4:1 was found for the permeability of K^+ and $Na^+ : Cl^-$, which indicates that PFM has higher conductance for monovalent cations than for Cl^- . In similar experiments with $CaCl_2$, a reversal potential of -4 mV was found, which indicates that the permeability ratio for $Ca^{++} : Cl^-$ is 1.2:1.

PFM activity in lipid bilayers was stable between pH 5 and 8. As the pH decreased below 5.0, even with low PFM concentrations, the conductance of the membrane

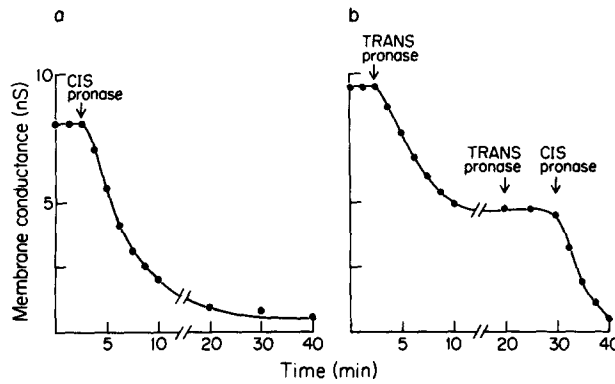


FIG. 10. Effect of pronase on PFM-treated membranes. Membranes were formed in buffer F, clamped at $+30$ mV, and treated with $50 \mu\text{g}$ of partially purified PFM. Pronase (1 mg/ml) was added 30 min after treatment with PFM. (a) Pronase was introduced through the *cis* side (same side as PFM). (b) Pronase added to the *trans* side and repeated 17 min later. More pronase was added to the *cis* side 10 min after the last addition.

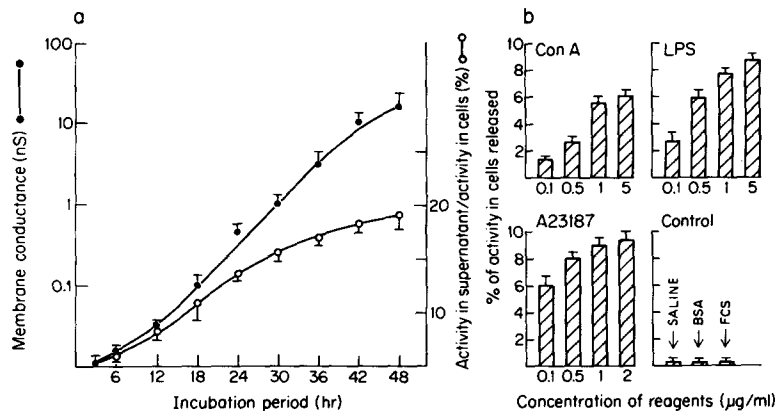


FIG. 11. (a) Secretion of PFM by viable *E. histolytica*. Amoebae (HM1, $2 \times 10^5/\text{ml}$) were washed three times with TYI-S-33 medium and incubated at 37°C . At intervals, 0.1 ml of the supernatant was tested for PFM activity in planar bilayers (in 0.1 M KCl , at constant $+30$ mV) and the conductance measured after 30 min of exposure (\bullet); activity was also given relative to that found in cells (washed three times with medium and freeze-thawed) (\circ). (b) Secretion of PFM induced by Con A, *E. coli* LPS, and A23187 as described in Materials and Methods. Saline (5%), bovine serum albumin (5 mg/ml), and fetal calf serum (5%) were used as controls. Data represent means of three experiments.

increased rapidly, which eventually resulted in membrane breakdown.

PFM Spans the Bilayer Membrane. We used pronase to investigate the transmembrane nature of PFM. Pronase added to the same (*cis*) side of PFM rapidly decreased the conductance to 6% of the initial value (Fig. 10 a). In contrast, pronase added to the *trans* side resulted in 50% inhibition; a second addition of pronase to the *trans* side had no effect (Fig. 10 b). However, further addition of pronase to the *cis* compartment resulted in nearly complete decrease in conductivity (Fig. 10 b). Pronase itself had no effect on membrane stability. This indicates that PFM has functional domains on both sides of the bilayer and that PFM inserts vectorially into the membrane.

Release of PFM by Viable Amoebae. The release of PFM into the extracellular medium by viable amoebae was examined. We observed pore-forming activity in the culture supernatant only after 6 h of incubation (Fig. 11 a). However, rapid stimulation of PFM release could be induced by membrane-active agents. Fig. 11 b shows the effect of A23187, a calcium ionophore, concanavalin A (Con A) and *E. coli* LPS on the release of PFM by viable amoebae after a 5 min incubation with the drugs. In each case, PFM was released from cells after stimulation and the amount of PFM did not increase over a 30-min period. However, the release induced by all three drugs was only 5–10% of the total amount of PFM contained in the cells (Fig. 11 b). Amoebae viability exceeding 98% was maintained in all experiments.

Effect of Serum and Serum Components on PFM Activity. Neither incubation with FCS (up to 30%) nor with fetuin (5 mg/ml) for 1 h (4°C, with agitation) before addition of PFM to planar bilayers had any inhibitory effect on pore-forming activity (data not shown).

Discussion

We describe a membrane-associated protein (PFM) from *E. histolytica* that forms aqueous pores in target membranes. In the presence of PFM, cells (macrophages and lymphocytes) rapidly depolarize, as measured by [³H]TPP⁺ equilibration technique. These changes in ionic permeability can be reproduced with protein-free lipid membranes, as we have shown for lipid vesicles by [³H]TPP⁺ uptake method and artificial membranes by the planar bilayer system. PFM behaves as an ion pore or channel since it satisfies the following criteria (19, 20): (a) PFM always conducts in defined discrete steps; (b) we could not resolve the transition between the conductance steps, even with a time resolution of 0.2 ms; (c) the magnitude of each conductance step (67 pS in 0.1 M KCl, which can be translated as an ion flux >10⁷ ions/s·channel) is considerably higher than that would be attained by active transport or carrier mechanism; and (d) PFM shows defined permselectivity ratios for cations/anions: it is four times more permeable to monovalent cations than anions and has slight preference (1.2 times) for Ca⁺⁺ over Cl⁻.

PFM appears to be a protein since it is pronase, trypsin, and heat labile. After insertion into a planar bilayer, PFM presents pronase-sensitive domains on both sides of the bilayer. The digestion with pronase on the same (*cis*) side of PFM is more effective, as it abolishes almost completely the conductance induced by PFM, in contrast to digestion by pronase on the opposite (*trans*) side of PFM, which only decreases the conductance by 50%. This indicates that PFM can insert into the bilayer, spanning the membrane in a vectorial fashion.

The linear relation (at low voltages) between PFM concentration and current flow

suggests that there is not a requirement for oligomers to be formed to conduct ions. However, we observed that, at low PFM concentration, some conductance jumps (135, 400, and 800 pS, which would correspond to dimers, hexamers, and dodecamers, respectively) were much more frequent than the unit step of 67 pS observed in 0.1 M KCl. This suggests a self-association of conducting monomers in the bilayer to form stable oligomers that are gated to open or close in a synchronous way.

PFM creates voltage-dependent channels in planar bilayers. The PFM channels are driven to a lower level of conductance by increasing the potential in both polarities across the membrane. This macroscopic voltage dependence can be explained at the level of single channels as a Poisson process, and the voltage dependence resides in the probability of finding the channels open or closed, rather than in a variable conductance of the channel itself. Similar observations have been made with other channel-forming proteins (21–23). A more quantitative description of the voltage-dependent behavior will be given elsewhere (J. D.-E. Young, T. M. Young, J. C. Unkeless, and Z. A. Cohn, manuscript in preparation).

It is not clear to us how PFM is related to the “toxic” factor (with M_r 25,000–45,000) from *E. histolytica* that exerts cytopathic effects on cultured nucleated cells only in serum-free medium (8, 24–27). PFM-enriched fractions (M_r 30,000 fraction) lyse rat and rabbit erythrocytes (J. D.-E. Young, unpublished observations) and can insert and create pores in planar bilayers in the presence of serum.

To examine the possibility that PFM may be released by amoebae after cell surface stimulation, we looked at release of PFM triggered by membrane-active agents. Amoebae do not release detectable amounts of PFM into the conditioned medium during 6 h of incubation. However, stimulation by A23187, Con A, and LPS from *E. coli* results in rapid release (within 10 min) of 5–10% of cell-associated PFM. These agents may mimic the interaction of amoebae with target cells and if so, the secretion into the limited intercellular spaces during effector-target contact may result in a much higher local concentration of PFM (allowing the formation of stable oligomers) and a correspondingly greater degree of target cell damage. These observations could explain the apparent discrepancy between the contact-dependent killing by live amoebae and the reversible, serum-inhibitable cytopathic effect of amoeba extracts on cultured cells (8, 24–27).

Alternatively, PFM may act in conjunction with other toxic factors released by amoebae and the channel formed by PFM might function as a conduit for small molecules from amoebae that would exert toxic activity only after being introduced into the target cell. Such transmembrane pathways for macromolecules have been described for bacterial outer membrane porins (28–30) and more recently for the B fragment of the diphtheria toxin, which presumably generates a channel large enough for the A fragment to pass through (31, 32). To test this possibility, experiments to determine the size of PFM channel are in progress in our laboratory.

We have also observed that PFM depolarizes *E. coli*, measured by [^3H]TPP $^+$ uptake (unpublished observations). It is possible that PFM may play a role in killing and ingestion of bacteria, which are an important source of food for amoebae. By inserting into the bacterial membranes, PFM may discharge the energized state of the bacteria, disrupting a number of active transport systems driven by the membrane potential. This may paralyze or kill the ingested bacteria in a way similar to the action of bacterial colicins (33).

Pore-forming activity has been found for a number of membrane-active antibiotics, secretory proteins, and toxins from bacteria (19, 20), and has been associated with the complement-mediated action on membranes (34-37). Among the bacterial pore-forming proteins, perhaps most noteworthy are the colicins secreted by bacteria. Colicins El, Ia, and K depolarize *E. coli* (38-40), and colicin K is known to create voltage-dependent channels in the planar bilayer membrane (23). This change in ion permeability induced by colicins is compatible with a single-hit killing mechanism for target bacteria (23, 33). The complement cascade also induces the formation of pores in the membrane and the colloid-osmotic lysis induced on cells by complement may be a direct consequence of membrane pore formation (34-37).

Thus, the PFM from *E. histolytica* may be a member of a general class of membrane-active macromolecular toxins produced and secreted by cells. Analogous pore-forming proteins that could be transferred to target cell membranes might participate in cytotoxicity mechanisms of other effector cells (macrophages, neutrophils, and lymphocytes) that mediate contact-dependent cytolysis. Moreover, the correlation between pore-forming activity in *E. histolytica* and virulence should also be examined.

During the course of these studies, the work of E. C. Lynch and C. Gitler was brought to our attention, who using whole cell lysates from *E. histolytica* have also found pore-forming activity in planar bilayers (manuscript submitted for publication).

Summary

We describe the partial purification and characterization of a pore-forming material (PFM) from *Entamoeba histolytica*. The formation of ion channels by PFM was examined in three systems. (a) PFM depolarizes J774 macrophages and mouse spleen lymphocytes as measured by [³H]TPP⁺ uptake. (b) PFM induces rapid monovalent cation flux across the membrane of phosphatidylcholine-cholesterol vesicles. (c) PFM confers a voltage-dependent conductance to artificial planar bilayers, which is resolved as a summation of opening of individually conducting steps of 67 pS in 0.1 M KCl. Monomers of PFM are functional; however, a preferential aggregation occurs in the planar bilayer. Activity is pronase, trypsin, and heat sensitive and is stable between pH 5-8. PFM is not secreted by unstimulated amoebae but after exposure to the calcium ionophore A23187, concanavalin A, and *E. coli* lipopolysaccharide, 5-10% of the total cell content of PFM is released into the medium within 5-10 min. High-performance gel filtration results in an ~1,000-fold purification of PFM and gives an M_r of 30,000. This protein may play a role in the cytotoxicity mediated by *E. histolytica*.

Note added in proof: Lynch et al. (41) have described a pore-forming protein from *E. histolytica*, which, under denaturing conditions, has a M_r of 13,000.

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