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Molecular Mechanisms of Cytotoxicity Mediated by *Entamoeba histolytica:* Characterization of a Pore-Forming Protein (PFP)

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Entamoeba histolytica is the human pathogen responsible for amebiasis. This infection is characterized by an invasive enteric illness that may spread to multiple organs. The parasite *E histolytica* may now be cultivated *in vitro,* with Diamond's axenic medium [1]. In culture, this protozoan remains cytolytic to a variety of cell types, including neutrophils and macrophages (reviewed in [2]). The mechanism(s) involved in the expression of this potent cytolysis remain(s) unclear. Previous studies from several laboratories have suggested that the cell killing mediated by amoeba is dependent on intimate contact of amoeba with the target cell membrane [3–9]. Following contact, the amoeba readily ingests the target cell (Fig. 1A). However, more recent cinemicroscopic and kinetic studies [8,9] (see also Fig. 1B) indicate that the cytolysis mediated by amoeba may occur prior to phagocytosis, raising the possibility of an extracellular cytolytic event triggered by surface contact.

Previous work from this laboratory has described the properties of a highly enriched plasma membrane fraction and surface polypeptides from axenically grown *E histolytica* [10,11]. Studies from this laboratory on its extensive vacuolar apparatus and its role in pinocytosis and intracellular acidification have also been published [12,13]. Here, we review some recent evidence for the isolation and characterization of a pore-forming protein (PFP) extracted from this protozoan, which may be closely related to the killing of target cells mediated by pore-forming proteins of the immune system (the complement cascade as well as proteins extracted from granules of different immune cells [Young et al, in preparation]), which may thereby suggest a very similar and general mechanism of cell-mediated cytotoxicity.

ISOLATION OF THE PORE-FORMING PROTEIN FROM *E HISTOLYTICA*

We were interested in isolating an active principle from amoeba lysates that could damage target cell membranes. In particular, we needed to design functional

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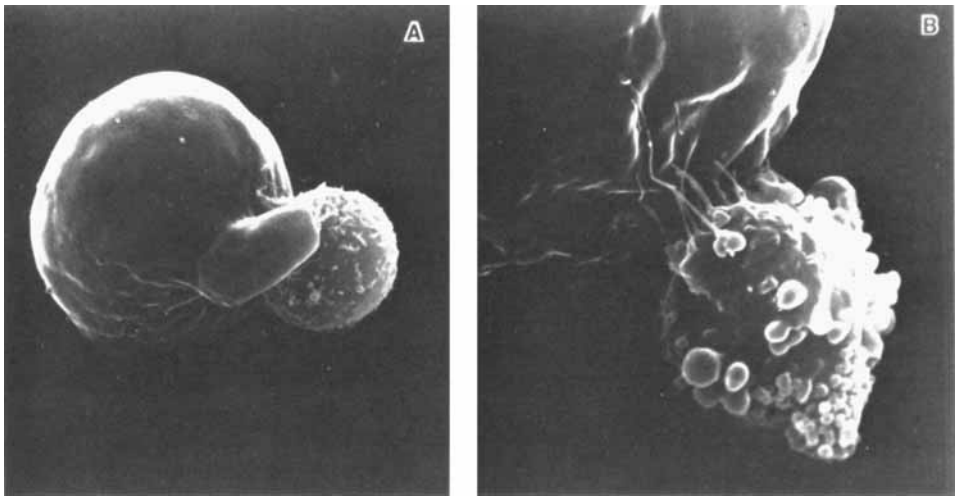

Fig. 1. Scanning micrographs of *E histolytica* trophozoites and J774 macrophages coplated on coverslips for 10 min. A) Initiation of phagocytosis of macrophage by amoeba, ×1,000. B) Attachment of macrophage to amoeba surface. The macrophage shows extensive membrane blebs, suggesting possible extracellular cytotoxic activity mediated by the amoeba, ×2,000. Macrophages attached to amoeba showing a blebbing surface are common findings at this early time point.

assays that could be used to screen and enrich for this membrane-damaging activity. A PFP produced by killer cells and presumably used to damage target cell membranes would be expected to change the transmembrane ionic balance in a dramatic way. First, at the whole cell level, PFPs should be able to depolarize the resting membrane potential of nucleated cells and to lyse anucleated erythrocytes. Lysis of erythrocytes occurs as a result of irreversible membrane pore formation and is due to lack of any appreciable membrane repair mechanism in those cells. At the level of model lipid membranes, putative PFPs can be assayed and examined more rigorously employing lipid vesicles and planar lipid bilayers. These systems provide clues to some molecular and biophysical properties associated with PFPs which would not be attained by other techniques.

We have shown that total amoeba lysates depolarize dramatically the membrane potential of macrophages and lymphocytes [14]. Similar depolarization effects have been found with *Fundulus* blastocells [16] and *E coli* spheroplasts [15]. Amoeba lysates also contain lytic activity to erythrocytes from various species [15,17]; this hemolytic activity is unstable and decays rapidly with time when lysates are stored at 4°C. This may be due to rapid loss of the activity produced by proteolytic enzymes known to be associated with amoeba lysates. Amoeba lysates have also been tested in lipid vesicles [14]. The strategy of our experiments consisted in loading lipid vesicles with different electrolytes. In the presence of amoeba PFP activity, cations become more permeable through lipid vesicle membranes than anions [14]. Such unequal transient distribution of ions across the membrane generates a negative intravesicular membrane potential that can be measured by equilibration of voltage-sensitive probes [14]. With this approach, we demonstrated that in the presence of amoeba lysates,

lipid vesicles become leaky to Na^+, K^+, and a number of other monovalent ions, and to Ca^{2+} to a lesser extent [14].

The most sensitive assay for PFPs is the planar bilayer system [14,18,19]. Here, defined lipids are used to produce high-impedance membranes that can be voltage-clamped and used for direct current measurements through the use of sensitive current amplifiers. The schematics of the membrane and the electrical connections of the bilayer system are shown in Figure 2. The lipid and buffer compositions in this system are well controlled, and the clamped transmembrane potential may mimic the plasma membrane potential of living cells. Because of the high impedance of the bilayer, one can measure small conductance changes with greater time resolution than with other techniques. Thus, the planar bilayer system provides insight into properties associated with individual molecules of PFPs. Using this technique, we showed that in the presence of amoeba lysates, there is a dramatic increase in membrane current that occurs in progressive incremental steps (Fig. 3). The increase in current in discrete steps may be interpreted as progressive and irreversible incorporation of units or groups of PFPs into the planar bilayer that then results in the opening of ion channels. The magnitude of each conductance step indicates an ion flux over 10^7 ions/sec/molecule, which is considerably higher than would be attained by active transport or carrier mechanism.

The PFP from different strains of *E histolytica* can be extracted from cells with the aid of detergent (β-D-octylglucoside [14,15]) or sodium dodecyl sulfate (SDS) [15,16] and assayed as described above. In the presence of β-D-octylglucoside, the PFP from cell lysates may be fractionated by high performance liquid chromatogra-

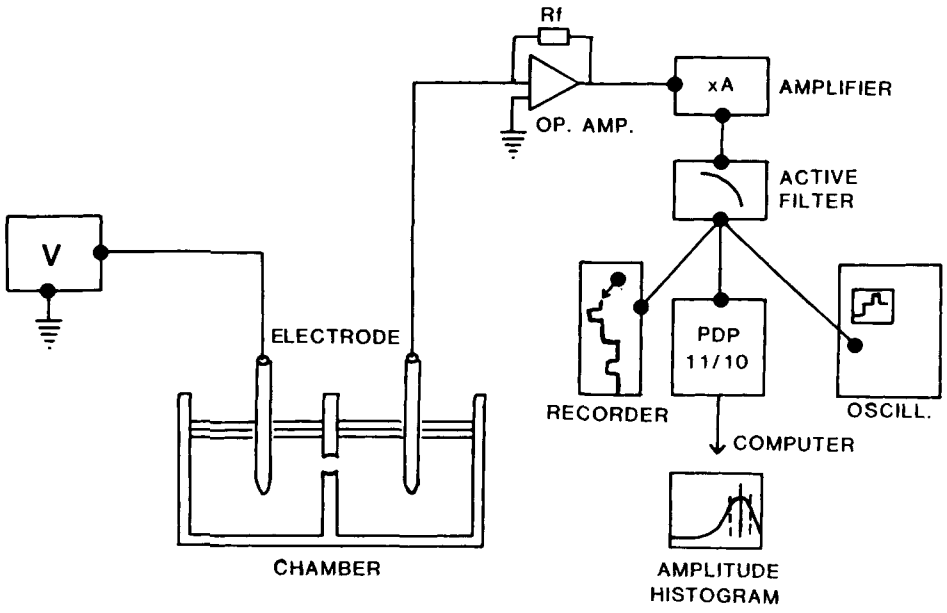

Fig. 2. Schematics of the planar bilayer system. The planar bilayer spans the hole across the two chambers. The transmembrane voltage is clamped by a DC battery. Current is measured via a pair of electrodes, and the signal is amplified by means of an operational amplifier. The output signal is then displayed on oscilloscope or chart recorder, and data are analyzed by hand or through the computer.

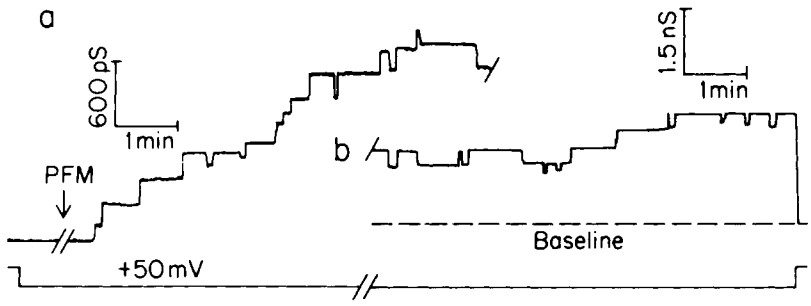

Fig. 3. Effect of amoeba extracts on planar bilayer membrane conductance. The bilayer was formed in symmetrical solutions containing 0.1 M KCl. The voltage across the membrane was clamped at +50 mV (the side of protein addition made positive). Pore-forming material (PFM, amoeba postnuclear supernatant, 1 μg protein) was added to the bilayer, followed by stirring (arrow, noise deleted). Note the increase in membrane conductance in discrete steps, indicating incorporation of single or groups of single channels into the bilayer. In (b), the record was continued at a different scale, after turning down the gain for the current.

phy (HPLC), under which conditions the activity is found to be associated with a M_r of 28–30 kilodaltons (kd) [14]. Surprisingly, even in the presence of β-D-octylglucoside, PFP readily aggregates with time, and when rechromatographed, may assume a higher M_r. Lysates fractionated by HPLC in the absence of any detergent show peaks of activity not only at the 28–30 kd region but also at much higher M_r's (> 200 kd). Activity in lysates is quickly lost even at 4°C in the presence of protease inhibitors. The initial activity decays by 40% after 4 hr and by 80% after 8 hr; for comparison, assay times in the planar bilayer typically take 15–25 min for completion. Upon treatment with 1% SDS, the protein assumes an electrophoretic mobility of 13–15 kd [15,16] (see Fig. 4A). As will be pointed out in more detail later, it is possible that this active species of 13–15 kd represents the monomeric species of PFP, and that other M_r's associated with the PFP activity reflect aggregation or oligomerization of the monomer. Interestingly, the material extracted by SDS maintains its pore-forming activity in planar lipid bilayers. Most of the pore-forming activity is associated with a subcellular membrane compartment [14,16], suggesting the possibility of PFP being localized in intracellular vacuoles.

We have recently extracted PFP employing buffers of low pH in the absence of any detergent, under which conditions the protein is solubilized and remains active (Fig. 4B). PFP extracted at low pH and fractionated by HPLC under acidic conditions show a fast migration mobility corresponding to a lower M_r species, but subsequent to dialysis against buffers with physiological pH and analysis by SDS-polyacrylamide gel electrophoresis (PAGE), the electrophoretic mobility assumes again that of a 14–15-kd protein (Fig. 4B). This pH-dependent difference in apparent M_r probably reflects a higher mobility (and thus lower apparent M_r) when the protein becomes soluble at low pH. The increased solubility at low pH also raises the intriguing possibility that if the PFP is indeed localized within acidic intracellular compartments, it may exist in a soluble form before being secreted into the intercellular space by amoeba. However, this inference still awaits definitive support from subcellular localization studies on PFP, which are currently being pursued in this laboratory.

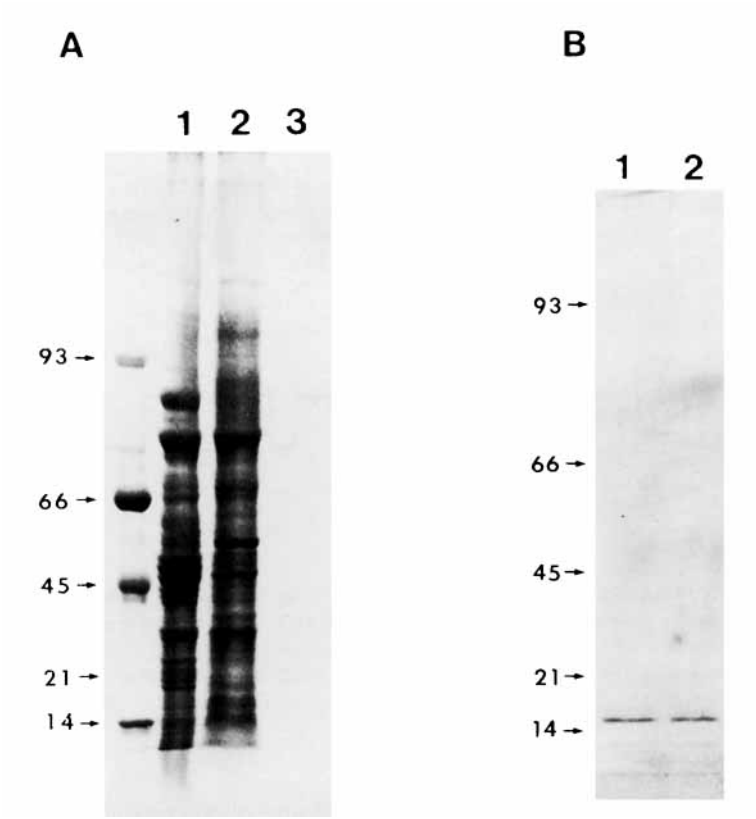

Fig. 4. Purification of the pore-forming protein from *E histolytica* lysates. SDS-PAGE (4–20% gradient). A) Lane 1) Total cell postnucleus lysate; 75 μg protein. Lane 2) Supernatant after centrifugation of lysate at 100,000g for 90 min, 4°C; 75 μg protein. Lane 3) Postmembrane supernatant after gel chromatography on S-200 columns and HPLC (TSK 3000); active fractions were assayed in planar bilayers; faint band corresponds to "active" material. B) Lanes 1,2) Proteins extracted from two different strains of amoebae employing ammonium acetate 0.2 M, pH 4.0, followed by Sephadex G-75 and TSK-3000 HPLC; active fractions were dialyzed to pH 7.0 and loaded on the gel.

The low-pH extraction procedure allows direct cytotoxicity assays in detergent-free conditions. The extracted PFP shows potent hemolytic activity [15]. Less than 100 ng of the enriched PFP is capable of lysing 10^8 erythrocytes in less than 10 min. In addition, preliminary experiments show that this material is capable of lysing nucleated cells (lymphocytes) [unpublished observations]. The lytic activity is not serum dependent, and in the presence of Ca^{2+}, lysis is only partially enhanced. The material aggregates at neutral pH, as evidenced by subsequent chromatography, and loses activity rapidly at pH 7.0.

BIOPHYSICAL PROPERTIES OF THE AMOEBA PFP

Single-channel events are observed when low amounts of PFP (0.1–1 ng) of protein are used (Fig. 5). Unit conductance steps of 67 pS (1 S = 1 A/1 V) are rarely observed. More frequently, we see doublets with a conductance of 135 pS, hexaplets of 400 pS, and dodecaplets of 800 pS, all of which turn "on" and "off" in synchrony

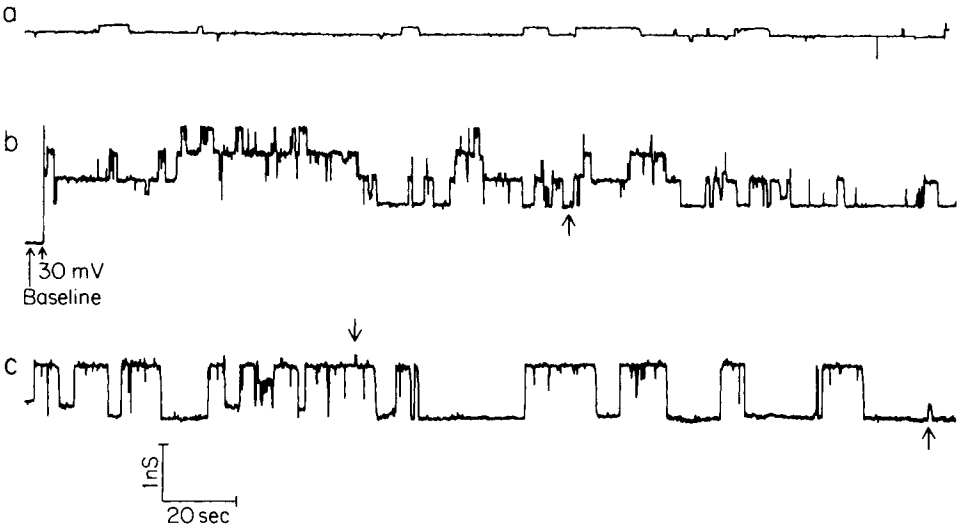

Fig. 5. Discrete conductance steps in planar bilayer induced by low amounts of pore-forming protein (10 pg). The membrane was made in buffer containing 0.1 M KCl, 10 mM Tris-Cl, pH 7.4, and clamped at +30 mV; the same scale was used for comparison. PFP was prepared by HPLC, as in [14]. a) Unitary steps of 67 picosiemens (pS) and doublets of 135 pS can be observed. b) Hexaplets of 400 pS, with less frequent smaller steps (such as the one pointed by arrow). c) Dodecaplets of 800 pS with smaller intermediate steps.

at low voltages. These results suggest that the PFP may oligomerize in the membrane to form larger conducting units, in much the same way as alamethicin [21]. These data are also consistent with our observations that PFP aggregates in the test tube, aasuming several M_r's by chromatography. However, oligomerization does not seem to be required for the conducting state of PFP since monomers are functional, as inferred from the double-logarithmic plot of conductance vs PFP concentration, which yields a slope of 1 [14].

The PFP from amoeba shows a remarkable voltage-dependent behavior. In planar bilayers containing large amounts of PFP, the application of a transmembrane voltage leads to instantaneous current flow, in much the same way as an ohmic resistor. However, at voltages higher than 30 mV (in both polarities), the initial current attained with the voltage step relaxes to a lower steady-state value with time. That is, the current flowing through the membrane at steady state increases linearly with the applied voltage, until a negative resistance region is reached (Fig. 6).

This peculiar voltage-dependent behavior can be explained at the level of single channels. We find that the current driven through each oligomeric unit increases proportionally with higher voltages (Fig. 7). However, the time for which the oligomer stays open is clearly voltage-dependent, the whole unit switching "off" more frequently with higher voltages. In addition, the oligomer disassembles at even higher voltages, so that the individual channels also close more often and behave independently at high voltages. It seems therefore that the macroscopic voltage-dependent conductance can be satisfactorily explained by parameters that describe single-channel kinetics.

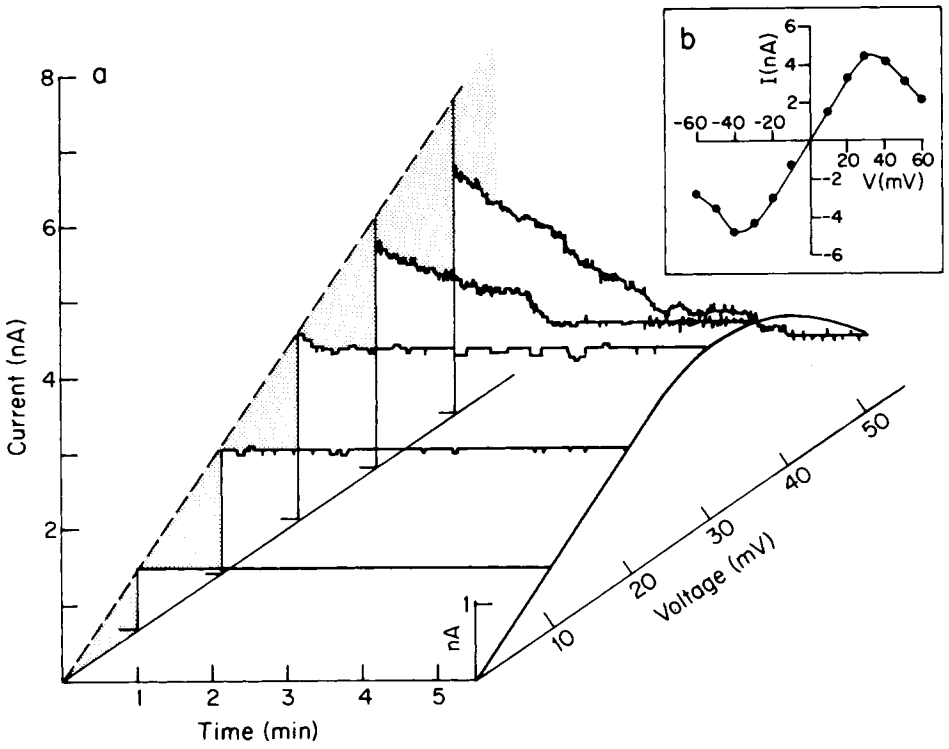

Fig. 6. The steady-state current-voltage plot of a membrane (in 0.1 M KCl) containing PFP. a) Voltage steps from 0 to +60 mV spaced at 10 mV intervals were applied across a membrane treated with 10 μg of partially purified PFP. The membrane current following the voltage pulse was allowed to relax for 5 min (note the time scale); at the end of this time interval, voltage across the membrane was turned to 0 for 1 min before next step. The value of current attained before turning off the voltage was used to generate the steady-state current-voltage plot seen in the inset b). PFP was prepared as described in [14].

Monovalent cations (K^+ and Na^+) are at least 3–4 times more permeable through the PFP-containing bilayers than Cl^- [14,16]. This result is inferred from the reversal potential to null current flow from a ten-fold higher KCl or NaCl concentration on one side of the membrane. Even Ca^{2+} appears to be relatively permeant through bilayers containing PFP.

PFP spans the bilayer membrane as pronases added to either side of the bilayer results in a decrease of membrane conductance associated with the pore-forming activity [14]. Thus, it can be inferred that PFP probably has functional domains on both sides of the bilayer.

PFP IS RELEASED BY LIVE AMOEBAE UPON SURFACE STIMULATION

The release of PFP into the extracellular medium by viable amoebae has been examined [14]. We observe pore-forming activity in the culture supernatant only after 6 hr of incubation (Fig. 8a). However, rapid stimulation of PFP release can be induced by surface-active drugs. Figure 8b shows the effect of A23187, a calcium ionophore, concanavalin A (Con A) and *E coli* LPS on the release of PFP by viable

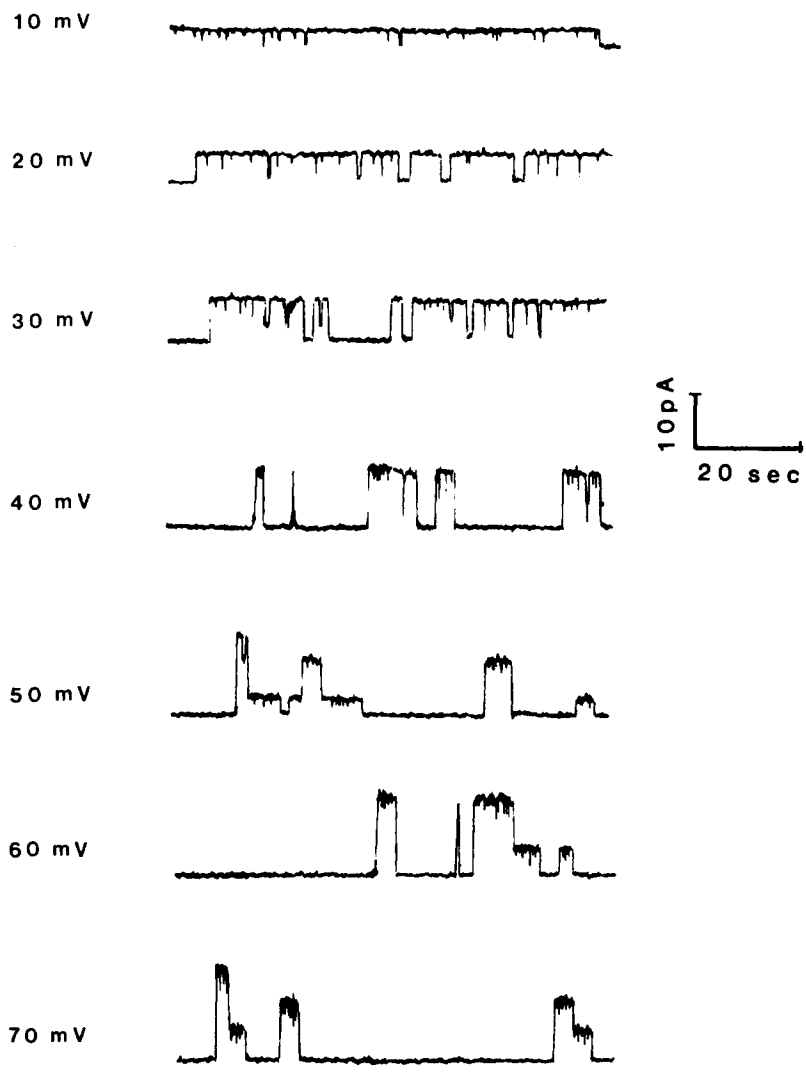

Fig. 7. Voltage-dependent conductance at the level of single channels. The membrane made in symmetrical 0.1 M KCl was treated with 5 pg of PFP, prepared as in [14]. The upward deflections represent openings of hexaplets at several different voltages. Note that the current driven through each hexaplet increases linearly with an increase in membrane voltage, while the opening time drecreases inversely with voltage. Note that at voltages over 50 mV, the unit starts to break down and smaller conductance steps may be seen to close and open independently.

amoebae after 5 min incubation with those agents. In each case, PFP is released from cells after stimulation, and the amount of PFP does not increase over a 30-min period. It is thus possible that other types of surface stimulation, such as cell-to-cell contact, may trigger similar release of PFP into closed intercellular spaces.

CONCLUSIONS

E histolytica cells are known to kill other cells by a contact-dependent mechanism. Killing by amoebae may involve a secretory event. A pore-forming protein

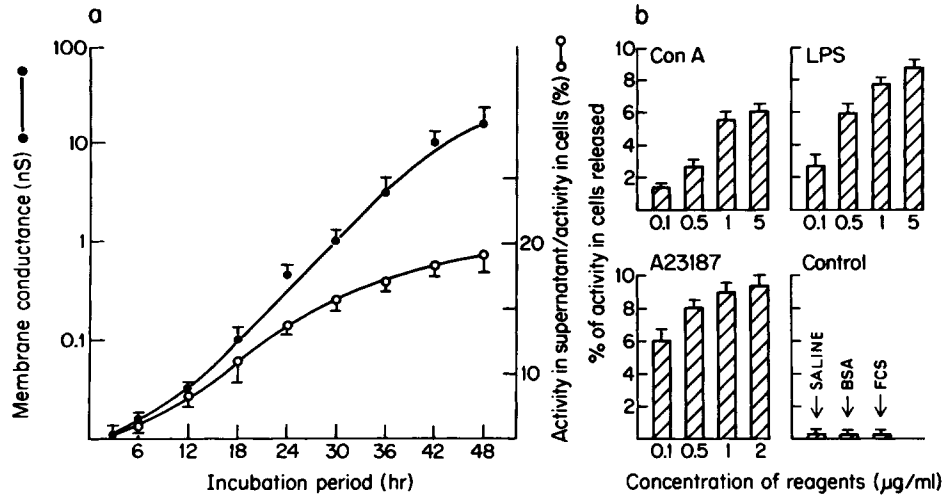

Fig. 8. a) Secretion of PFP by viable *E histolytica*. Amoebae (strain HMI, 2 × 10^5/ml) were washed three times with medium and incubated at 37°C. At intervals, 0.1 ml of the supernatant was tested for PFP activity in planar bilayers and the conductance measured after 30 min of exposure (○); activity was also given relative to that found in cells (●). b) Secretion of PFP induced by Con A, *E coli*, LPS, and A23187. Saline (5%), bovine serum albumin (5 mg/ml), and FBS (5%) were used as controls. [From ref. 14]

(PFP) from amoebae has been extracted, and its properties have been studied by means of several different techniques. It is not clear to us how PFP is related to the "toxic" factor (with M_r of 25–45 kd) from *E histolytica* that exerts cytopathic effects on cultured nucleated cells only in serum-free medium [8,22–25]. The PFP described by us lyses erythrocytes and inserts and creates pores in lipid bilayers in the presence of serum. PFP is released from amoebae following stimulation by surface-active agents. Such type of stimulation may mimic the interaction of amoebae with target cells, and if so, the secretion into the diffusion-limited intercellular spaces during effector-target contact may result in a much higher local concentration of PFP (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 [8,22–25]. The precise role of PFP in the cytotoxicity mediated by virulent amoebae, if any, remains to be established. By disrupting the transmembrane ionic balance of target cells and allowing a Ca^{2+} influx into these same damaged cells, such action may result in an osmotic-colloid type of cytolysis. This proposed mechanism of injury mediated by amoebae would be expected to occur in a vectorial direction; that is, the cytolytic PFP assembles only in target membranes, and if so, the question of why amoebae themselves stay viable remains unanswered.

The PFP from *E histolytica* may represent a member of a more 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 that mediate contact-dependent cytolysis.

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