Membrane Channel Formation by the Lymphocyte Pore-forming Protein: Comparison between Susceptible and Resistant Target Cells

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Abstract. The assembly of pores by the pore-forming protein (perforin) of cytolytic T lymphocytes (CTLs) and natural killer cells on the membranes of different cell lines was studied. Using the patch clamp technique in the whole cell configuration, we measured the conductance increase induced by perforin in susceptible cell lines as well as in resistant CTL lines (CTLLs). The results showed that although the amplitudes of the first observed conductance steps produced in both cell types were comparable, CTLLs required at least 10-fold higher doses of perforin to form membrane pores. Outside-out patches excised from CTLL-R8, on the other hand, appeared to be more susceptible to channel formation by perforin than intact cells, as lower doses were able to induce conductance increases. Once channels were induced in CTL membranes, however, their conductances (>1 nS) were indistinguishable from the ones obtained in susceptible cell lines. Fluorescence measurements with quin-2 showed that perforin induced rapid increases in the intracellular Ca^{2+} concentration in susceptible EL4 cells. In marked contrast, a perforin dose 60–120-fold higher than the minimal dose required to elicit Ca^{2+} changes in EL4 cells was not able to induce any measurable Ca^{2+} increase in CTLL-R8. The data suggest that the resistance of CTLs to lysis mediated by their own mediator perforin is at least in part due to their ability to avoid pore formation by this protein. The mechanism underlying this phenomenon is not yet understood, but the observation that outside-out patches excised from CTLL-R8 are more susceptible to channel formation by perforin than intact cells raises the possibility that an intracellular mechanism may be involved.

CYTOTOXIC T lymphocytes (CTLs)[1] and natural killer cells kill target cells by a mechanism that, according to one widely proposed model, is mediated by secretion of granule contents which include among other mediators a pore-forming protein (perforin or cytolysin) structurally related to the terminal components of the complement cascade (7, 16, 18, 25, 29). Purified perforin inserts into cell membranes and planar lipid bilayers in a Ca^{2+}-dependent fashion, forming nonselective ion pores that can be detected by standard electrophysiological techniques (1, 29, 30). The homopolymers formed by perforin resemble tubules with internal diameters of 15–20 nm. In contrast with proteins of the complement system, perforin binds directly to lipid bilayers, abrogating the need for interaction with other soluble proteins.

CTLs and natural killer cells are themselves highly resistant to killing mediated by other effector lymphocytes (3, 14, 23), their own granules (3, 27), and perforin itself (9, 12, 15, 22). The mechanism responsible for this self-protection is not understood. At least two possibilities may be postulated to explain this resistance phenomenon. When challenged with perforin, resistant cells may eliminate or somehow inactivate perforin pores after they are formed or, alternatively, protection may be conferred at an earlier stage, e.g., at the level of membrane binding/insertion preceding perforin polymerization and pore formation. To address this issue we felt it was necessary to use techniques that could be used to study the membrane permeability changes with a time resolution much better than that of conventional cytotoxicity assays, such as the ^{51}Cr-release assay used in most previous studies. Here, we report observations made using the patch clamp technique and quin-2 fluorescence measurements, and show that early conductance changes and an increase in intracellular free Ca^{2+} concentration is readily induced by perforin in susceptible cells but not in resistant CTLs. These findings suggest that the perforin-resistance mechanism of CTLs is at least in part confined to the stages of membrane binding and/or insertion during the assembly of membrane lesions by perforin.

Materials and Methods

Perforin Purification

Perforin was purified to homogeneity (tested by silver stain and amino terminal sequencing [17]) from granule-enriched fractions from the CTL line

1. *Abbreviations used in this paper*: CTL, cytotoxic T lymphocyte; CTLL, CTL line; HU, hemolytic unit.

(CTLL)-R8 after chromatography through DEAE-Sepharose, Q-Sepharose, Polyanion SI, and Superose 12 columns (Pharmacia Fine Chemicals, Uppsala, Sweden) as previously described (9, 17), and concentrated finally on a Mono-Q column. One hemolytic unit (HU) is the dose required to lyse 50% of 10^7 sheep red blood cells in a standard assay (9). Perforin used in this work was either homogeneous (60–120 HU/μl) or partially purified from a Polyanion SI column (30 HU/μl). No differences were observed between the two samples throughout our experiments. The sample buffer contained 1 mM EGTA, 20 mM Tris-HCl, pH 7.2, and either 700 mM NaCl (Polyanion) or 500 mM NaCl (Mono-Q).

Cells

All cells were kept at 37°C in a humidified atmosphere containing 5% CO_2 until use in experiments. The perforin-susceptible cell lines EL4 (mouse T cell lymphoma), Rl-1 (mouse lymphoma), K562 (human erythroleukemia), NIH-3T3 (mouse fibroblast), and P815 (mouse mastocytoma) were maintained in RPMI-1640 (Gibco Laboratories, Grand Island, NY), 5% FBS, bicarbonate, 50 U/ml penicillin, and 50 μg/ml streptomycin. CTLL-R8 and CTLL-2 were maintained in alpha MEM supplemented as above, plus 10% IL-2-containing leukocyte-conditioned medium. The susceptibility and resistance of the various cell lines to perforin were established in other experiments using a standard ^{51}Cr release assay performed in RPMI-1640 in the absence of serum, as described (9, 12, 15). CTLL-R8 and CTLL-2 are resistant to purified perforin in doses up to 700 HU/ml.

Electrophysiological Measurements

Whole cell and outside-out patch clamping was performed at room temperature using standard techniques (6). Cells were transferred to the culture chamber built into the stage of an inverted microscope equipped with Nomarski optics and kept in 800 μl RPMI medium containing 0.1% BSA and 10 mM Hepes, pH 7.4. This medium contains 0.42 mM Ca^{2+}, a concentration shown in preliminary studies to be sufficient for perforin-mediated lytic activity. The cell morphology was better preserved in this medium as compared to simpler buffers without BSA. The presence of 0.1% BSA did not interfere with the formation or stability of gigaohm seals in the cells used in this study. An EPC-7 amplifier (List Electronics, Darmstadt, FRG) was used to perform the voltage clamp and current measurements. Pipette tips were coated with Sylgard and heat polished as described (6). Current and voltage were simultaneously registered on a paper chart recorder and recorded at 5 KHz bandwidth on an FM tape recorder for future analysis. The pipette solution contained 155 mM K-glutamate, 5 mM NaCl, 2 mM $MgCl_2$, 10 mM K-Hepes, pH 7.2, and either 0.1 mM or 10 mM K_2EGTA. No differences were observed between these two EGTA concentrations. Perforin was diluted in water immediately before use in a volume calculated to make the sample isotonic and was carefully added to the cells. The isotonic buffer alone did not induce any conductance changes in the cell membranes. Adding perforin during an electrophysiological recording posed some restrictions on the accuracy at which the final dose at the cell membrane could be determined. This is in part due to the fact that perforin inactivates quickly in the presence of Ca^{2+} (7, 18, 25, 29).

Quin-2 Measurements

CTLL-R8 and EL4 cells were loaded with quin-2/AM, and the intracellular Ca^{2+} content was estimated essentially as described (26). In brief, quin-2/AM (Molecular Probes Inc., Eugene, OR) was solubilized in DMSO and added to 2×10^7 cells/ml to a final concentration of 20 μM quin-2 and 0.2% DMSO. After an incubation period of 30–60 min in RPMI medium containing 5% FBS, cells were washed three times in the experimental solution (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Hepes, pH 7.4), diluted to $2–5 \times 10^6$ cells/ml and used within 1 h. Another wash was performed after 30 min to eliminate interference from spontaneous leakage of the fluorescent probe (26). Experiments were performed with cell suspensions at room temperature (25°C) with a fluorescence spectrophotometer (model 203; Perkin-Elmer Corp., Norwalk, CT) (excitation 339 nm, emission 490 nm). Ca^{2+} levels were determined using the formula

$$(Ca^{2+})_i = (K_D)\ (F\text{-}F_{min})/(F_{max}\text{-}F),$$

where F is the experimental fluorescence level measured. At the end of each recording, Triton X-100 was added to the cell suspension to a final concentration of 0.05% and the fluorescence measured was taken as F_{max}. F_{min} is the fluorescence obtained after subsequent addition of EGTA to the lysed cells to a final concentration of 10–20 mM. The K_D value was taken as 115 nM (26). In control experiments addition of EGTA to the cell suspension before lysis with Triton X-100 gave no significant fluorescence reduction, indicating that the amount of extracellular quin-2 and, hence, leakage was not detectable. Perforin was added to cell suspensions at several final concentrations after dilution in water to make the solution isotonic. To facilitate mixing after addition of perforin, cells were gently aspirated up and down two times for ~5 s using a tubing. Addition of control buffer containing no perforin to cell suspensions did not induce any detectable increase in the fluorescence level (not shown).

Results

Conductance Response in Perforin-susceptible Cells

Fig. 1 *A* shows membrane currents recorded from an EL4 cell after perforin was added as indicated. EL4 cells are known to be highly susceptible to attack by perforin (9). An inward current developed after addition of perforin which increased slowly to >10 nA. In other similar experiments (not shown) it was found that this inward current reversed direction at ~0 mV, indicating that the current flows through a membrane pathway without significant ion selectivity. Fig. 1 *B* shows a magnified view of the segment marked by an arrow in Fig. 1 *A*. As the inward current increased, the trace became progressively noisier. In previous work in which perforin was applied to pure lipid bilayers (1, 29, 30), the protein resulted in stepwise conductance increases, with step amplitudes ranging from 0.4 to 6 nS. These conductance steps observed in lipid bilayers were attributed to nonselective ion channels formed by perforin. In Fig. 1, *A* and *B* most of the increase in inward current was not obviously stepwise. However, closer inspection shows that the inward current always started with a current step that had a rise time of a millisecond or less (uppermost trace in Fig. 1 *C*). Of nine EL4 cells challenged with 30 HU of perforin, three gave responses as in Fig. 1. Similar results were also obtained in two of the three cells that were challenged with 15 HU/ml (the smallest dose tried).

When larger amounts of perforin were applied (60–150 HU), recordings similar to those in Fig. 1 were obtained in all but one of 22 EL4 cells. The initial conductance steps were most frequently observed within 5–30 s after addition of perforin. Current steps recorded at a holding potential of −60 mV were converted into conductances. The average conductance from first steps was 1.68 ± 0.18 nS (n = 12), and from first and subsequent steps 1.54 ± 0.11 nS (n = 26). Responses similar to Fig. 1 were recorded also from all four 3T3-fibroblasts (30–150 HU, conductance step amplitude of 1.68 ± 0.20 nS, n = 6) and on three of four outside-out patches pulled from 3T3 fibroblasts (150 HU, 1.64 ± 0.20 nS, n = 13, values ± SEM). Since these means are statistically indistinguishable, all conductance steps observed in EL4 cells and fibroblasts were collected in the histogram of Fig. 1 *D* (grand mean 1.59 ± 0.09 nS, median 1.45 nS, n = 45; conductance changes which did not start as steps [rise times < 1 ms] were not considered here). The values observed here are comparable to those found in lipid bilayers (1, 29, 30).

In all but 1 of 26 experiments on EL4 cells and fibroblasts, the conductance increase started with a step. Often, the initial step was the only step that could b edetected, and the subsequent increase in inward current occurred more gradually. Conductance changes as in Fig. 1 were also obtained with other plasma membrane holding potentials (−60 to +20 mV).

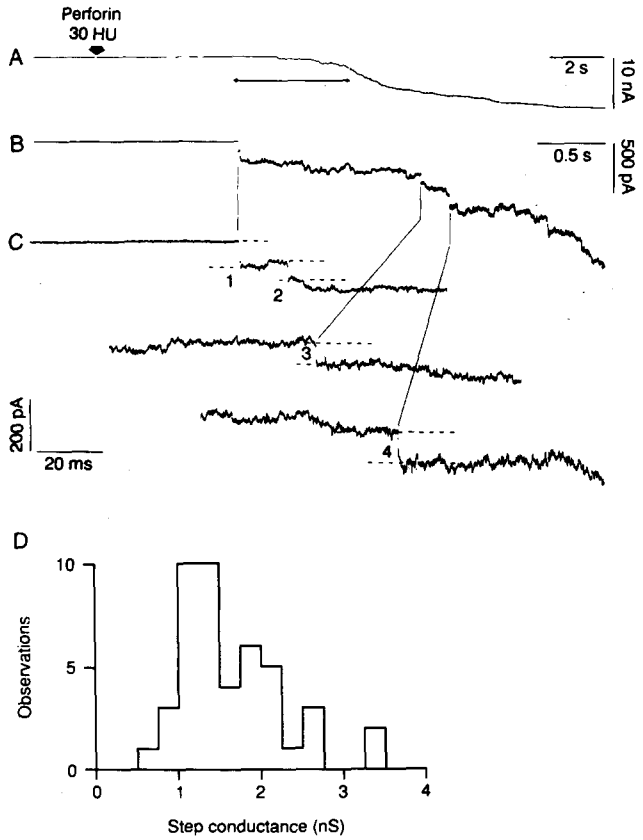

Figure 1. Conductance increase in perforin-susceptible cells. (*A*) Whole cell recording of membrane current from an EL4 cell. The trace starts <2 min after establishing a connection between the pipette and the cytosol. 30 HU of partially purified perforin in 5 μl of saline was applied as indicated to 800 μl of bathing cell medium. At uniform dilution, the perforin concentration would be 37.5 HU/ml. Holding potential was −60 mV. (*B*) Magnified view of the portion in *A* delineated by a horizontal arrow. Current steps are indicated by three continuous lines that point to traces in *C* where the sections containing the steps are magnified further. Four steps were detected in *C*; their amplitudes were measured by fitting horizontal lines to traces as indicated (*dashed*). (*D*) Histogram of conductance steps obtained as illustrated in *C*. The histogram includes whole cell measurements from EL4 and NIH-3T3 cells, and from outside-out patches pulled from NIH-3T3 fibroblasts. Perforin was applied <3 min after establishing connection with the cytosol. Holding potential was −60 mV throughout.

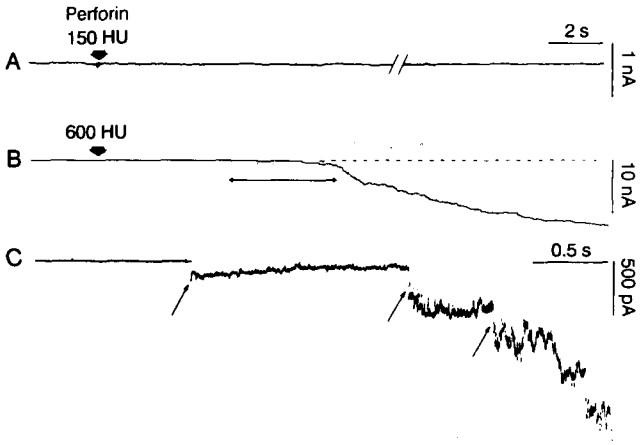

Figure 2. Currents recorded from two CTLL-R8 cells after application of 150 (*A*) and 600 HU (*B*). In *A*, a 150-s interval was excised from the record; no conductance increase was observed during that time. Traces *A* and *B* start in <2 min after establishing connection with the cytosol. (*C*) Magnified view of a section in trace *B* (marked by a *horizontal arrow*). Two current steps were detected (*arrows*) during playback at higher speed. Hold potential was −35 mV in *A* and −60 mV in *B* and *C*. Perforin was partially purified in *A* and purified to homogeneity in *B*.

Perforin-induced currents at 20 mV were outward (not shown). Evidently pore formation by perforin is not strongly potential sensitive. Inward currents with characteristics similar to those observed with EL4 and 3T3 cells were recorded also when 150 HU perforin was added to other susceptible cells (K562, Rl-1, and P815).

Conductance Response in Perforin-resistant Cells

Fig. 2 *A* shows an experiment similar to that of Fig. 1 *A* conducted on a perforin-resistant cell line (CTLL-R8). Although the amount of perforin added to the chamber was five times higher than that in Fig. 1, no conductance increase was observed in this nor in any of the other nine experiments where 150 HU perforin was applied within 5 min of establishing a connection between the pipette and the cytosol. The same experimental conditions were used to study another resistant cell line, CTLL-2, and, again, no conductance increase was observed in any of the six cells studied (not shown). We performed two experiments with even larger (600 HU) amounts of perforin added. One experiment produced no conductance change, while the other did (Fig. 2 *B*). The inward current seen in this experiment was similar to that in Fig. 1 *B*. It is shown magnified in Fig. 2 *C*; the trace contained three rapid conductance steps (*arrows*).

Subsequent experiments showed that CTLL-R8 cells may lose some of their resistance to perforin when their cytoplasm is removed or disturbed. For instance, 5 out of 12 outside-out patches pulled from CTLL-R8 cells responded with conductance steps to 150 HU. One of these records is shown in Fig. 3 *A*; note that this is in marked contrast to whole cell records obtained on CTLL-R8 under similar conditions. For comparison, Fig. 3 *B* shows an outside-out patch record obtained on a perforin-susceptible cell line, 3T3, which also displays insertion of perforin pores of comparable amplitudes. Since even in whole cells the cytoplasm is slowly dialyzed against the solution in the recording pipette while recording is in progress, we asked whether cells that have been clamped for longer periods of time became more susceptible to perforin. When we recorded from whole CTLL-R8 cells for >7 min before applying perforin, some responded to 150 HU perforin with conductance steps. Further experiments are required to explore the possibility that cytosolic factors may contribute to the resistance of CTLL-R8 cells to perforin. Nonetheless, conductance steps in four whole CTLL-R8 cells were 1.1 ± 0.11 nS, $n = 6$, and in six outside-out patches clamped at −60 mV the amplitude was 1.41 ± 0.31 nS, $n = 16$. These values are comparable to those observed in EL4 cells and 3T3 fibroblasts.

Although the perforin concentrations near the cells in Figs. 1 and 2 could not be accurately determined (see legend to Fig. 1 and Materials and Methods), it seems reasonable

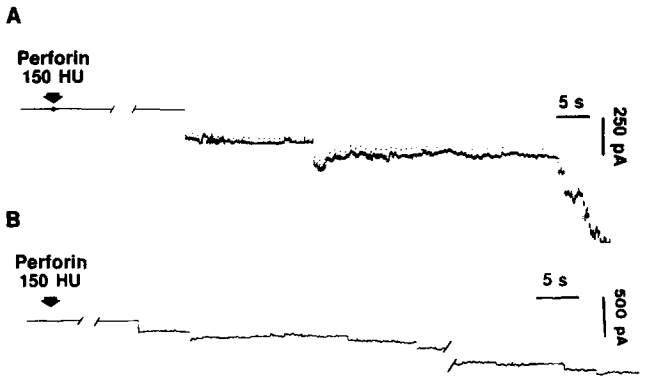

Figure 3. Currents recorded from outside-out patches of resistant and susceptible cells. (*A*) A patch of CTLL-R8 after application of 150 HU of partially purified perforin following the procedures of Fig. 2. The record starts after 30 s in the whole cell configuration and 270 s after excising the patch. A 30-s interval was excised from the record. No conductance change was observed during that time. Holding potential was −60 mV. (*B*) A patch of 3T3 cell treated with 150 HU of partially purified perforin. As indicated by bars, two intervals were excised from the record: 50 s after addition of PFP (*left*) and 30 s during conductance changes (*right*).

to conclude that the increase in membrane conductance requires higher perforin concentrations in CTLL-R8 cells than in EL4 cells and fibroblasts.

Cell Blebbing

When perforin (15–150 HU) caused pore formation in an EL4 cell or a 3T3 fibroblast, the surrounding cells always responded with pronounced morphologic changes (blebbing, see Fig. 4, *A* and *B*). The resistant cells CTLL-R8 (Fig. 4, *C–E*) and CTLL-2 (not shown) did not bleb unless an extremely high perforin concentration was used (e.g., 600 HU in the experiments of Fig. 2). In intact cells, therefore, blebbing correlated well with pore formation, consistent with the idea that CTLL-2 and CTLL-R8 cells are protected against lysis because they do not readily form pores.

Although outside-out patches from CTLL-R8 cells frequently showed pore formation in response to 150 HU perforin (Fig. 3 *A*), blebs failed to appear in the surrounding cells in all of 12 such experiments. In contrast, during similar experiments performed with outside-out patches of the susceptible cell line 3T3, the surrounding cells were seen to bleb, usually just after pore detection in the patches. Together, these observations are consistent with the notion that the outside-out patches of CTLL-R8 may be less resistant to perforin than intact CTLL-R8.

Calcium Measurements

To further verify the possibility that pore formation by perforin is restricted in CTL membranes, we took advantage of the fact that these pores are permeable to Ca^{2+} (29), and measured the intracellular free Ca^{2+} concentrations of EL4 and CTLL-R8 cells in suspension before and after addition of perforin. In this series of experiments, homogenization of the cell–perforin suspension could be achieved in the first 5 s after addition of perforin, overcoming the difficulties with determining the final effective dose used. The mean values of the intracellular free Ca^{2+} concentrations of resting cells were 62 ± 12 nm (n = 10) for EL4 cells and 82 ± 16 nm (n = 9) for CTLL-R8 cells. As shown in Fig. 5, addition of perforin at doses as small as 15 HU/ml was able to induce measurable increases in Ca^{2+} concentrations in EL4 cells. The effect of perforin was dose dependent and reached saturating levels of fluorescence at ~60 HU/ml, a dose usually high enough to induce maximum cell killing as assayed in a standard ^{51}Cr release assay (references 9, 12, and 15 and data not shown). The fluorescence increase reached a plateau within the first 4–5 min after addition of perforin at all doses >15 HU/ml (Fig. 5 *A*). The level of the Ca^{2+} signal remained high for at least 20 min, and showed only a small decrease consistent with the spontaneous decay in the quin-2 fluorescence, as determined in control experiments using cells permeabilized by Triton X-100 (not shown). In marked contrast with the EL4 results, CTLL-R8 cells did not show measurable increases in Ca^{2+} concentration immediately (Fig. 5 *B*) or 15 min (not shown) after addition of doses as high as 600 HU/ml. Except for one out of the three experiments in which 7.5 HU/ml of perforin was added to EL4 cells, all other higher doses used showed increases in Ca^{2+} concentrations as shown in Fig. 5 *C*. In the experiments using CTLL-R8, however, no indication of intracellular Ca^{2+} increase was detected after addition of perforin in doses ranging from 75 to 900 HU/ml (Fig. 5 *C*). The above results show that a dose at least 60–120-fold larger than the minimal one required to induce a measurable Ca^{2+} concentration increase in susceptible cells may be necessary to induce the same effect on the membrane of CTLL-R8 cells. The Ca^{2+} measurements described above were performed using quin-2 as the Ca^{2+} probe; similar results were obtained by using fura-2 as the calcium indicator (data not shown).

Standard ^{51}Cr release assays were next used to quantitate the cytotoxicity levels produced by the various doses of perforin used here. Data are shown only for EL4 and CTLL-R8 cells in Fig. 6. In general, the extent of lysis correlated well with an increase in intracellular free Ca^{2+} concentrations. It should be noted that both CTLL-R8 (Fig. 6 *A*) and CTLL-2 (not shown) were resistant to purified perforin in doses up to 700 HU/ml. CTLL-R8 cells showed 10–20% lysis at 3,500–7,000 HU/ml, a dose range ~100-fold higher than that needed to induce the same effect in susceptible cell lines.

Discussion

The resistance of cytolytic lymphocytes to lysis mediated by their own cytotoxic protein, perforin, has been described (3, 9, 14, 22, 27), but the underlying mechanism is still unknown. Here, we investigated the first events that occur at target cell membranes immediately after addition of perforin, and compared the results obtained on perforin-susceptible target cells with those on cytolytic lymphocytes known to be resistant to this pore-forming toxin. Our main observations are: (*a*) perforin induces large conductance pores on membranes of susceptible cells within a few seconds after its addition to the culture medium; (*b*) in the following 0.5–3 min of cell blebbing an increase in the intracellular Ca^{2+} concentration can be observed; and (*c*) neither of these effects is easily detected in perforin-resistant cytolytic lymphocytes. Our results confirm and extend previous experi-

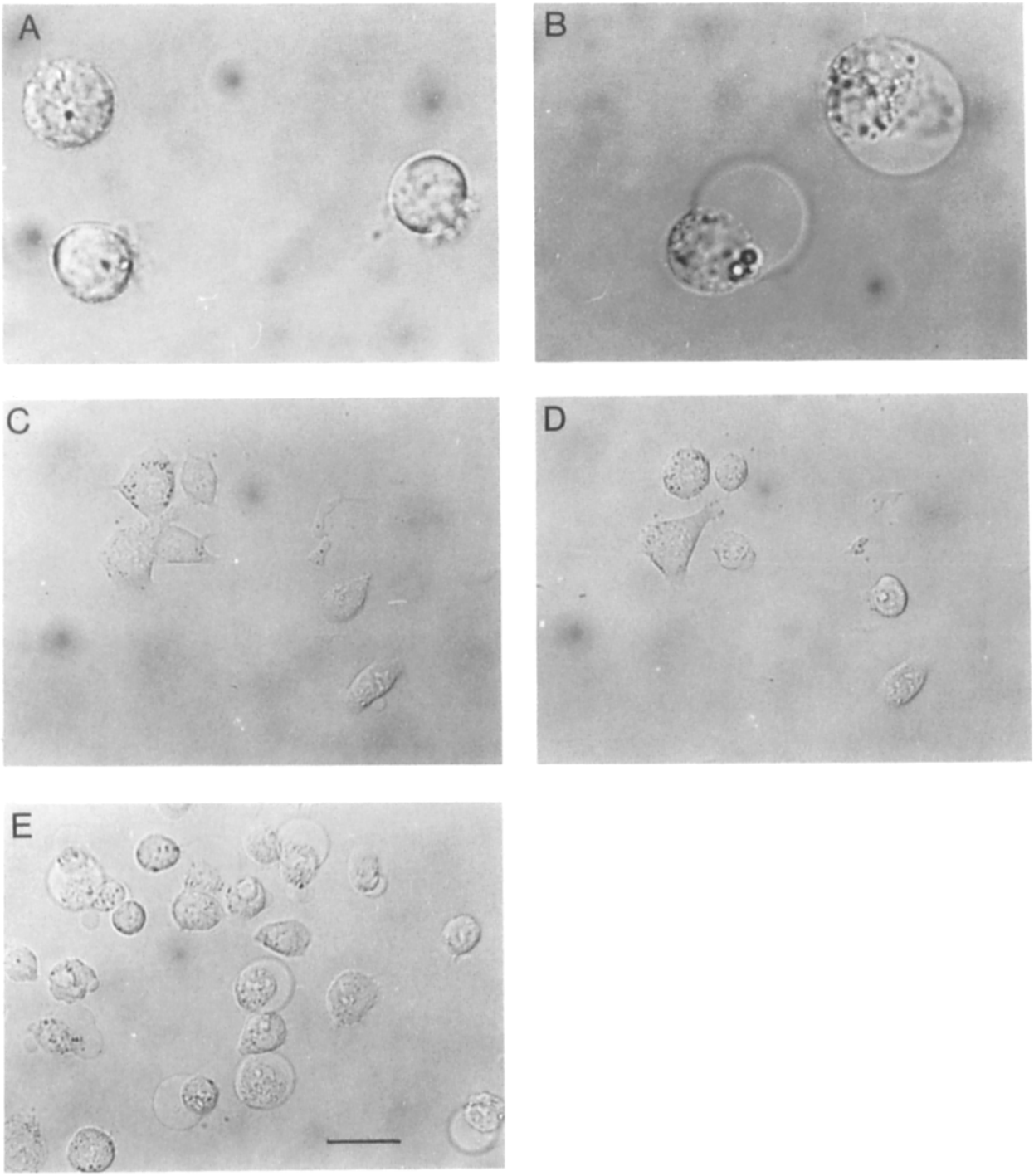

Figure 4. Cell morphology as observed under the microscope during electrophysiological measurements. EL4 cells are shown before (*A*) and 4 min after (*B*) the addition of 150 HU of purified perforin. Typical blebbing was observed in <1 min. The same microscope field containing CTLL-R8 cells is shown before (*C*) and 5 min after (*D*) the addition of 150 HU perforin. No blebbing was observed in either this cell or any other cell in the culture chamber. (*E*) CTLL-R8 cells 7 min after addition of 600 HU perforin close to the microscope field. Cell blebbing observed in <1 min after addition of perforin was restricted to the region surrounding the field shown. Perforin was either partially purified (*B* and *D*) or purified to homogeneity (*E*). Bar: (*A* and *B*) 8 μm; (*C–E*) 20 μm.

ments performed on susceptible cells (1, 29, 30), and suggest that avoiding pore formation may be one of the first events that determine the perforin-resistance phenotype of cytolytic lymphocytes.

We conclude that perforin-induced lesions begin with the sudden opening of an aqueous pore of ~1.5 nS conductance. Occasionally, a few other similar pores open later, but usually the subsequent increase in plasma membrane conductance occurs in increments too small to be detected as steps on the increasingly noisy traces. We suggest that the relatively gradual conductance increase is in some way a consequence of preceding conductance steps. Perhaps a perforin

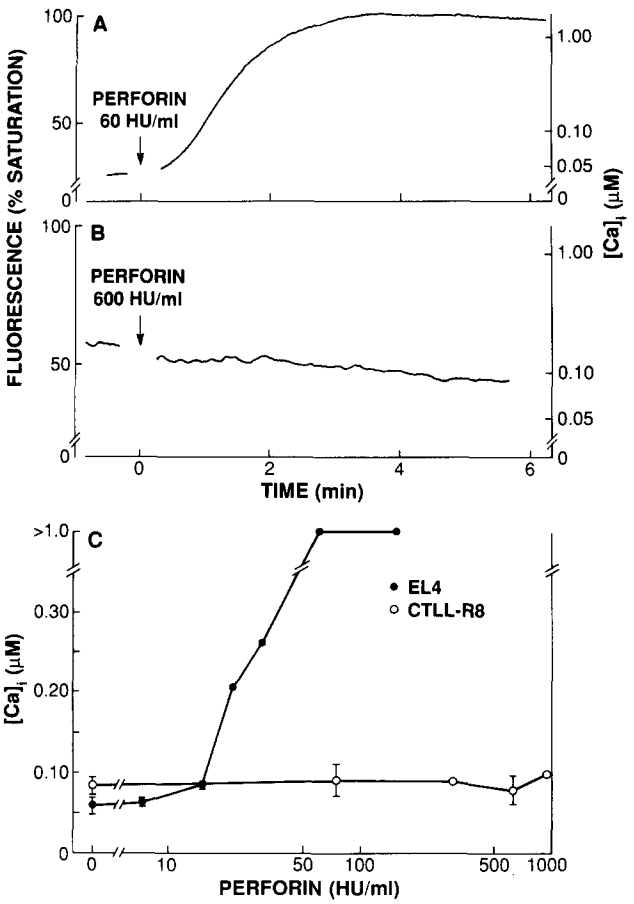

Figure 5. Quin-2 fluorescence measurements after treatment of EL4 and CTLL-R8 cells with perforin. (*A*) The fluorescence increases to saturating level after adding 60 HU/ml perforin to EL4 cells. (*B*) No significant Ca^{2+} increase is detected after adding 600 HU/ml to CTLL-R8 cells. The apparent reduction of fluorescence level after addition of perforin was attributed to a dilution of cell concentration due to the excess volume of perforin-containing buffer that needed to be introduced, as determined in control experiments (not shown). (*C*) Dose-effect curve obtained after adding perforin to EL4 (●) and CTLL-R8 cells (○). Error bars show ± SEM. Points without error bars represent single measurements unless they indicate values >1 μM. For the ordinates in *A* and *B*, 0% saturation represents F_{min} and 100% F_{max} (see Materials and Methods). Ca^{2+} concentration was calculated according to the formula described in Materials and Methods. Perforin was either partially purified (EL4 and CTLL cells) or purified to homogeneity (CTLL-R8, two highest doses).

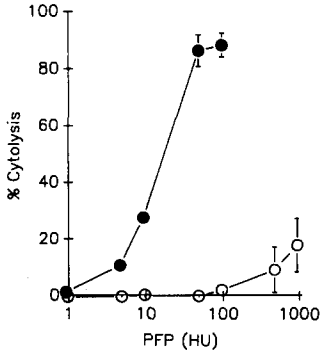

Figure 6. (*A*) Lysis of susceptible and resistant cells by perforin. Perforin-mediated lysis of EL4 (●) and CTLL-R8 (○) cells was measured in a 3-h ^{51}Cr-release assay, and the percent of cytolysis was calculated as described (9, 12). Each point represents the average of triplicate determinations.

pore, once open, can be joined and enlarged by other perforin monomers. Alternatively, the large Ca^{2+} influx expected through a perforin pore may lead to the opening of endogenous, Ca^{2+}-activated ion channels in the plasma membrane. Ca^{2+}-activated channels that are nonselectively permeable to small cations (including Ca^{2+}) have been observed in neutrophils (28).

The magnitudes of the conductance steps observed in susceptible cells were comparable to the ones obtained with resistant cell membranes, suggesting that once perforin reaches a certain threshold dose, the pores formed in the two types of membranes are similar, consistent with the view that the protection mechanism acts before pore formation.

The exact concentration of perforin at the cell membrane could not be estimated precisely in our experiments. Nevertheless, considering that we could observe pore formation and blebbing in EL4 cells using doses of 15 HU (two out of three cells) and that we have never observed these changes in CTLLs when as much as 150 HU of perforin was applied (nine CTLL-R8 and six CTLL-2 cells), we can roughly estimate that the threshold for pore formation in the two CTLLs we studied is at least 10-fold larger than the one for susceptible EL4 cells. The minimal dose of perforin required to induce 10–20% lysis of CTLL-R8 cells, as determined by the ^{51}Cr release assay, is ~100 times larger than the dose required to induce a similar degree of lysis in a variety of susceptible cell lines (reference 12 and Fig. 6), a value larger than the 10-fold increase required for pore formation. It is not clear at this moment whether such differences can be ascribed only to difficulties in determining the effective perforin dose in the patch clamp experiments. More experiments are required to clarify this point and to establish the relationship between the number of pores formed per cell and the degree of cytotoxicity. Our estimates indicate that the amount of perforin produced by a single cytolytic lymphocyte may be sufficient to lyse a target cell, since 20 HU of perforin, produced by 4×10^4 lymphocytes, induces maximal ^{51}Cr release from 5×10^4 target cells (our unpublished observations).

The quin-2 experiments shown in Fig. 5 clearly demonstrate that pore formation by perforin in susceptible cells is accompanied by a sustained increase of intracellular Ca^{2+} levels. This result is not surprising since perforin pores show high conductance values and little selectivity for small ions, as we (29, 30) and others (1) have observed. These results also suggest that perforin may be directly involved in inducing the intracellular Ca^{2+} increase in target cells that is usually associated with a lethal hit produced by cytolytic lymphocytes (19, 24). The minimal dose of perforin required to elicit a Ca^{2+} response in EL4 cells was ~7.5–15 HU/ml, while doses up to 900 HU/ml (60–120-fold higher) were unable to induce detectable Ca^{2+} increases in CTLL-R8. Since the doses of perforin used in these Ca^{2+} measurement studies are more accurately determined than the electrophysiological experiments and correlate more closely with values found in ^{51}Cr-release experiments (reference 9 and Fig. 6), it is suggested that perforin-induced increases in intracellular free Ca^{2+} concentration may be used as a convenient assay for cell killing.

Although the mechanism underlying the perforin resistance phenomenon is not understood, it is tempting to draw analogies with the complement system, since perforin and

the various terminal complement components are structurally and functionally homologous. In the complement system, a protein (called homologous restriction factor) has been identified and shown to be in part responsible for protecting cells against lysis mediated by complement of the homologous species (20, 21, 31). Homologous restriction factor has also been shown to protect lymphocytes against perforin-mediated lysis (32, 33). However, studies from our laboratory and others (13) have clearly demonstrated that homologous restriction factor does not in fact play any role in restricting perforin-mediated cytolysis. Other candidate molecules or mechanisms must be sought instead to explain this resistance phenomenon.

One other hypothesis that may explain the resistance phenomenon is that the insertion of the initial pores into cell membranes could trigger an intracellular process (enhanced endocytosis or membrane healing?) leading to the inactivation/elimination of the pores as well as conferring resistance to cell membranes against subsequent pore insertions. Our data showed, however, that such "initial" lesions are not formed at all by perforin in resistant cell membranes at doses that are highly effective on susceptible cells. Other investigators have reported on "reversible" lesions formed by perforin in Lettre cells (1), but this reversion phenomenon was not observed by us either in the whole cell–clamp experiments (Fig. 1) or in the quin-2 measurements (Fig. 5).

Our results with outside-out recordings (Fig. 3) suggest that perforin pores are more easily inserted into excised membrane patches than into whole CTLL-R8 cells. This conclusion is also supported by the morphological observation that no cell blebbing could be observed in neighboring cells present in the microscope field, indicating that, at the dose used, pore formation was confined to the excised patch of membrane. More experiments are needed to clarify this point and to investigate the possibility that at least part of the protection mechanism may require a specific intracellular component. If this turns out to be the case, it may represent an important adaptation for prevention of cell lysis in a situation in which the lipid moiety of the membrane offers an almost unlimited number of insertion sites for the pore-forming toxin. As pointed out earlier, we have consistently observed a difference in the amount of perforin required to form pores in whole cell patch experiments versus that required to lyse cells or to induce calcium fluxes. That is, it takes much less perforin (10-fold difference) to induce conductance changes. It is possible that during whole cell recordings the continued dialysis of the intracellular medium may rapidly deplete the cell of the putative intracellular resistance-conferring component, thereby decreasing the cell resistance to perforin. This and any other speculations will have to be substantiated by more definitive studies.

Finally, it should be pointed out that self-protection of cells against their own toxins is not limited to lymphocytes (2). Yeasts, for example, are also known to produce a pore-former killer toxin that lyses susceptible target cells but not the toxin-producing yeasts themselves (4). The resistance phenotype in yeast is thought to be conferred by an "immunity" protein encoded by the same gene that encodes for the killer toxin (4). Colicin-resistant bacteria are also known to produce a protective protein (5). It is possible that protective mechanisms similar to the one used by lymphocytes may be generally applicable to a variety of other killer cells. The use of techniques that give a fast resolution time at the level of cell membrane may continue to give important information which should help us obtain better clues on this type of killing mechanism.

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