Aerolysin is a pore-forming toxin that plays a key role in the pathogenesis of *Aeromonas hydrophila* infections. In this study, we have analyzed the effect of aerolysin on human granulocytes (HL-60 cells). Proaerolysin could bind to these cells, was processed into active aerolysin, and led to membrane depolarization, indicating that granulocytes are potential targets for this toxin. Fura-2 measurements were used to analyze the effect of aerolysin on cytosolic [Ca\(^{2+}\)] homeostasis. As expected for a pore-forming toxin, aerolysin addition led to Ca\(^{2+}\) influx across the plasma membrane. In addition, the toxin triggered Ca\(^{2+}\) release from agonist and thapsigargin-sensitive intracellular Ca\(^{2+}\) stores. This Ca\(^{2+}\) release was independent of the aerolysin-induced Ca\(^{2+}\) influx and occurred in two kinetically distinct phases: an initial rapid and transient phase and a second, more sustained, phase. The first, but not the second phase was sensitive to pertussis toxin. Activation of pertussis toxin-sensitive G-proteins appeared to be a consequence of pore formation, rather than receptor activation through aerolysin-binding, as it: (i) was not observed with a binding competent, insertion-incompetent aerolysin mutant, (ii) had a marked lag time, and (iii) was also observed in response to other bacterial pore-forming toxins (staphylococcal α-toxin, streptolysin O) which are thought to bind to different receptors. G-protein activation through pore-forming toxins stimulated cellular functions, as evidenced by pertussis toxin-sensitive chemotaxis. Our results demonstrate that granulocytes are potential target cells for aerolysin and that in these cells, Ca\(^{2+}\) signaling in response to a pore-forming toxin involves G-protein-dependent cell activation and Ca\(^{2+}\) release from intracellular stores.

### EXPERIMENTAL PROCEDURES

**Materials—**Cell culture media were obtained from Life Technologies, Inc. (Paisley, Scotland), U73122 from Calbiochem (La Jolla, CA), and DISC(6), PBFI, and fura-2/AM from Molecular Probes (Eugene, OR). All other chemicals were purchased from Sigma or Fluka. The "Ca\(^{2+}\)-free medium" contained: 143 mM NaCl, 6 mM KCl, 1 mM MgSO\(_4\), 5.6 mM glucose (0.1%), 20 mM HEPES pH 7.4, and 0.1 mM EGTA. The "Ca\(^{2+}\)-free medium" consisted of Ca\(^{2+}\)-free medium supplemented with 1 mM CaCl\(_2\).

**Culture of HL-60 Cells—**HL-60 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, penicillin (50 units/ml), streptomycin (50 μg/ml), and L-glutamine (2 mM) at 37°C in a humidified atmosphere of 5% CO\(_2\), 95% air. Granulocytic differentiation was initiated by addition of dimethyl sulfoxide (Me\(_2\)SO) (final concentration 1.3% for 3 days, then 0.65% for 1 or 2 days).

The abbreviations used are: GPI, glycosylphosphatidylinositol; PAGE, polyacrylamide gel electrophoresis; PLC, phospholipase C; Ins(1,4,5)P\(_3\), inositol 1,4,5-trisphosphate; Me\(_2\)SO, dimethyl sulfoxide; ER, endoplasmic reticulum; fMLP, fMet-Leu-Phe; PBFI, K\(^{+}\)-binding benzofuran isophthalate.

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Proaerolysin Purification—Wild type and variant proaerolysins were purified as described previously (19). Concentrations were determined by measuring the optical density (O.D.) at 280 nm, considering a 1 mg/ml sample has an O.D. of 2.5 (20). Proaerolysin was labeled with 125I using IODO-GEN reagent (Pierce) according to the manufacturer's recommendations. Proaerolysin was incubated with this footage of a chemical probe and this fragment of bovine serum albumin. We consistently obtained a specific activity of about 2 x 10^9 cpm/µg of proaerolysin. 125I-Proaerolysin ran as a single band on an SDS gel. Aerolysin was obtained by treating proaerolysin with trypsin at a protein to enzyme ratio of 1/20 (mol:mol) for 10 min at room temperature. After which a 10-fold excess of trypsin inhibitor was added.

Proaerolysin Binding Experiments—HL-60 granulocytes at a concentration of 2 x 10^6 cells/ml in Ca^2+ medium were incubated with 125I-proaerolysin for 25 min at 4 °C, spun down in a table top cell centrifuge and washed once and resuspended in a medium containing Ca^2+ and Mg^2+. The last washing step was performed twice. In competition experiments, 125I-proaerolysin and the unlabeled wild type or mutant toxin were added to the cells simultaneously. For SDS-PAGE analysis, cells were briefly sonicated with a tip sonicator in sample buffer. SDS-PAGE was performed as described by Laemmli (21).

Membrane Potential Measurements—HL-60 granulocytes were washed once and resuspended in buffer containing 20 mM HEPES pH 7.4, 4 mM KCl, 1 mM EGTA, 3 mM CaCl_2, 1 mM MgSO_4, 1 mM CaCl_2, 0.5 mM bovine serum albumin, and 0.25 mM sulfonpyrazone, to a final density of 2 x 10^6 cells/ml (22). Cells were incubated with the cell-permeant form of the K^+-binding benzofuran isophthalate dye PBFI-AM (stock solution in Me_2SO in presence of pluronic acid F-127, final concentrations of 5 µM PBFI and 0.02% F-127) for 30 min at 37 °C, followed by 30 min at room temperature, washed once and resuspended in the same buffer, in the absence of bovine serum albumin, to a final density of 2 x 10^6 cells/ml. Fluorescence measurements were performed using a Photon Technology International fluorometer. The excitation and emission wavelengths were 343 and 460 nm, respectively. Single fluorescent traces were expressed as the ratio I/I_0 max, i.e., fluorescence intensity at a given time over maximal fluorescence intensity.

Measurements of Intracellular K^+-Concentration—HL-60 granulocytes were washed once and resuspended in loading medium, containing 20 mM HEPES pH 7.4, 5.6 mM glucose, 143 mM NaCl, 6 mM KCl, 1 mM MgSO_4, 1 mM CaCl_2, 0.5% bovine serum albumin, and 0.25 mM sulfonpyrazone, to a final density of 20 x 10^6 cells/ml (22). Cells were incubated with the cell-permeant form of the K^+-binding benzofuran isophthalate dye PBFI-AM (stock solution in Me_2SO in presence of pluronic acid F-127, final concentrations of 5 µM PBFI and 0.02% F-127) for 30 min at 37 °C, followed by 30 min at room temperature, washed once and resuspended in the same buffer, in the absence of bovine serum albumin, to a final density of 2 x 10^6 cells/ml. Fluorescence measurements were performed using a Photon Technology International fluorometer. The excitation and emission wavelengths were 343 and 460 nm, respectively. Single fluorescent traces were expressed as the ratio I/I_0 max, i.e., fluorescence intensity at a given time over maximal fluorescence intensity.

Measurements of Intracellular Ca^2+ Concentration—HL-60 granulocytes were washed once and resuspended in 20 mM HEPES pH 7.4, 5.6 mM glucose, 143 mM NaCl, 6 mM KCl, 1 mM MgSO_4, 1 mM CaCl_2, 0.5% bovine serum albumin, and 0.25 mM sulfonpyrazone, to a final density of 20 x 10^6 cells/ml (22). Cells were incubated with the cell-permeant form of the Ca^2+-inducer fura-2. Cells (2 x 10^6/ml) suspended in Ca^2+ medium containing 0.1% bovine serum albumin were loaded for 45 min at 37 °C with 2 µM fura-2/AM, then diluted to 10^3/ml and kept on ice. Just before use, a sample of loaded cells (2 x 10^6/ml) was centrifuged and resuspended in the desired medium. Fluorescence measurements were performed on a Perkin-Elmer fluorometer (LS5, Perkin-Elmer), thermostated at 37 °C. Excitation and emission wavelengths were 340 and 505 nm, respectively. Calibration was performed for each cuvette by sequential addition of 2 mM CaCl_2 (for Ca^2+-free medium), 1 µM ionomycin to measure Ca^2+ saturated fura-2 (F_0 max), followed by 24 mM EGTA, 75 mM Tris, pH 9.3, and 0.1% Triton X-100 to measure Ca^2+ free fura-2 (F_0 min). A relatively small leakage of fura-2 occurred in cells exposed to aerolysin (see “Results”). Results are shown as relative fura-2 fluorescence, normalized with respect to the maximal fluorescence (=100%) and minimal fluorescence (=0%) values obtained through the calibration procedure.

Measurement of Mn^2+ and Ni^2+ Entry—At an excitation wavelength of 360 nm, fura-2 fluorescence is Ca^2+ independent, the fluorescence of the probe is, however, quenched by several divalent cations. In this study, we used fura-2 to study entry of Mn^2+ and Ni^2+ in response to aerolysin independently from changes in [Ca^2+]. Cell-associated fluorescence before addition of the respective divalent cation was defined as 100% fluorescence. For the quantitation of the Mn^2+ influx at different times after aerolysin addition, we proceeded as described previously (23). Briefly, the percentage of fluorescence quenching that is immediately reversible after addition diethylenetriaminepentaacetic acid. Under these conditions, the fraction of the Mn^2+-induced fluorescence quenching that is immediately reversible after addition diethylenetriaminepentaacetic acid is a direct measure of extracellular fura-2 (23).

Chemotaxis Assay—For the chemotaxis assay, a Transwell chemoattractant chamber (6.5 mm diameter, 3 µm pore size, Corning Costar Corp., Cambridge, MA) was used. In this system, the cell reservoir (=upper chamber) is separated from the target chamber (=lower chamber) by a microporous membrane. The cell reservoir contained 10^6 cells in 100 µl of Ca^2+ buffer with 0.1% bovine serum albumin. The target chamber contained the indicated concentration of chemotax attractant or the appropriate solvent control in 500 µl of Ca^2+ buffer with 0.1% bovine serum albumin. Chemotaxis was allowed to occur over a period of 90 min in an CO_2 (5%) and temperature (37 °C) controlled incubator. Cells in the target chamber were counted. Results are expressed as cells recovered in the target chamber (% of cells that were initially added in the cell reservoir).

RESULTS

Binding to HL-60 Cells—As a first step in the characterization of the interaction of proaerolysin with myeloid cells, we have investigated whether proaerolysin was able to bind to HL-60 promyelocytes and HL-60 granulocytes. Cells were incubated with 1 nM proaerolysin at 4 °C for 10 min, washed, sedimented, and analyzed by SDS-PAGE followed by Western blot analysis for the presence of the toxin. Both wild type proaerolysin as well as a double cysteine mutant, G202C/I445C (see below), were able to bind to both types of HL-60 cells (not shown).

To investigate whether binding of proaerolysin was specific, we have analyzed whether radiolabeled and unlabeled proaerolysin could compete for binding to HL-60 cells. Binding of radiolabeled proaerolysin (4 nM) to both promyelocytic and granulocytic HL-60 cells could be inhibited by more than 80% by the presence of a 100-fold excess of unlabeled toxin (0.4 µM), indicating the presence of a limited number of binding sites. Binding of radiolabeled wild type toxin could also be inhibited, to the same extent, by unlabeled G202C/I445C mutant toxin, indicating that both forms of the toxin bind to the same sites.

These observations suggest that aerolysin binds to a limited number of sites on HL-60 cells. Using a previously described proaerolysin overlay assay (14), we could identify 4 proaerolysin-binding proteins (not shown). Binding to these proteins could be inhibited by 70% by treating the cells with the phosphatidylinositol-specific phospholipase C indicating that these putative receptors were GPI anchored (not shown). These four proteins remain to be identified. We could, however, exclude that binding occurred within 1 min after Mn^2+ addition was determined. The relatively small fraction of the fluorescence quenching that was due to the presence of extracellular fura (see below) was subtracted. The emission wavelength was 505 nm.

Determination of Extracellular Fura-2—To determine the amount of extracellular fura-2, we exposed fura-2-loaded cells for various times to aerolysin, followed by the addition of 25 µM Mn^2+ and, 1 min later, 100 µM of the heavy metal chelator diethylenetriaminepentaacetic acid. Under these conditions, the fraction of the Mn^2+-induced fluorescence quenching that is immediately reversible after addition diethylenetriaminepentaacetic acid is a direct measure of extracellular fura-2 (23).

Wild type and variant proaerolysins were purified as described previously (19). Concentrations were determined by measuring the optical density (O.D.) at 280 nm, considering that a 1 mg/ml sample has an O.D. of 2.5 (20). Proaerolysin was labeled with 125I using IODO-GEN reagent (Pierce) according to the manufacturer's recommendations. Proaerolysin was incubated with this footage of a chemical probe and this fragment of bovine serum albumin. We consistently obtained a specific activity of about 2 x 10^9 cpm/µg of proaerolysin. 125I-Proaerolysin ran as a single band on an SDS gel. Aerolysin was obtained by treating proaerolysin with trypsin at a protein to enzyme ratio of 1/20 (mol:mol) for 10 min at room temperature. After which a 10-fold excess of trypsin inhibitor was added.
Aerolysin Induces Plasma Membrane Depolarization in HL-60 Granulocytes—To investigate whether HL-60 granulocytes are sensitive to aerolysin, we have analyzed the effect of the toxin on membrane potential using the fluorescent probe DiSC₃(5) which has been widely used for this purpose (25). As shown in Fig. 1A, proaerolysin led to depolarization of the granulocytes with kinetics that were dose-dependent. As suspected, a marked increase in the rate of depolarization was observed when activating the protoxin prior to the addition to the cells (Fig. 1B). As shown in Fig. 1C, depolarization was in part due to the efflux of K⁺. As a control, we tested the hemolytically inactive mutant of aerolysin, G202C/I445C. This mutant contains two engineered cystein residues that form a disulfide bridge between the propeptide and the mature toxin (20). Even after trypsin activation, this mutant is unable to lyse erythrocytes presumably because it cannot oligomerize. Also G202C/I445C did not induce K⁺ efflux from HL-60 granulocytes. Surprisingly, depolarization of HL-60 granulocytes as well as K⁺ efflux followed two-step kinetics for reasons that remain to be established. In contrast, kinetics of membrane depolarization induced by the thiol-activated toxin streptolysin O were monophasic (not shown).

The observed K⁺ efflux and membrane depolarization were not due to lysis of the cells as illustrated by the fact that most cells still exclude ethidium homodimer-1 after 10 min (Fig. 1D). Faster kinetics of entry were observed with the smaller dye DiSC₃(5) which has been widely used for this purpose (25). As shown in Fig. 1D, aerolysin induced influx in ethidium homodimer-1 (full line). Trypsin-activated aerolysin was added at the time indicated by an arrow. D, aerolysin induced influx in ethidium bromide (dashed line) and ethidium homodimer-1 (full line). Trypsin-activated aerolysin was added at the time indicated by an arrow.

Fig. 3. Aerolysin Induces Plasma Membrane Depolarization in HL-60 Granulocytes. A and B, changes induced by proaerolysin (A) and trypsin-activated aerolysin (B) of the fluorescence of the membrane potential-sensitive probe DiSC₃(5) after uptake by HL-60 granulocytes. C, the aerolysin induced change in intracellular K⁺ was followed by monitoring the changes of PBF1 fluorescence after loading the cells with this dye as described under “Experimental Procedures.” Trypsin-activated aerolysin was added at the time indicated by an arrow. D, aerolysin induced influx in ethidium bromide (dashed line) and ethidium homodimer-1 (full line). Trypsin-activated aerolysin was added at the time indicated by an arrow.

These observations show that proaerolysin and aerolysin are able to bind to HL-60 cells and that the cells express proteases that can process the protoxin to its mature form. This allows heptamerization of the toxin and channel formation thereby leading to efflux of intracellular potassium, presumably to concomitant sodium entry, and membrane depolarization.

Aerolysin Induces [Ca²⁺]c Elevations Which Display Complex Kinetics—To investigate whether the interaction of aerolysin with myeloid cells leads to changes in cytosolic free Ca²⁺ concentration ([Ca²⁺]c), we exposed fura-2-loaded HL-60 promyelocytes and HL-60 granulocytes to either proaerolysin or trypsin-activated aerolysin (Fig. 3). Both, the protoxin and the mature toxin induced elevation of [Ca²⁺]c in a dose-dependent manner and this in both cell types. The kinetics of the [Ca²⁺]c increase were, however, markedly faster when the mature toxin was added rather than its precursor as previously observed for membrane depolarization (Fig. 3, A and C versus B and D). Also, the effects of both the pro and the mature toxin were more pronounced on the differentiated granulocytic HL-60 cells than on the immature promyelocytic cells. No changes in [Ca²⁺]c could be observed upon addition of the hemolytically inactive G202C/I445C mutant when added either in the pro or the mature form (Fig. 3D).

A striking feature of the aerolysin-induced [Ca²⁺]c elevations was their complex kinetics. Rather than presenting a monophasic increase, as might be expected for the insertion of a pore into the membrane, the kinetics were multiphasic: an initial, albeit at far slower rate, we investigated whether HL-60 granulocytes expressed proteases able to process the protoxin. As shown in Fig. 2, although the protoxin added to the cells showed no sign of contamination by aerolysin (lane a), a lower molecular weight form corresponding to aerolysin could be observed upon interaction with the granulocytes. A higher molecular weight band could also be observed upon incubation at 37 °C corresponding to the aerolysin heptamer (Fig. 2). These results agree well with our previous observations that proaerolysin can be converted into aerolysin by proteases provided by the host cell (14)².

² Abrami, M. Fivaz, and F. G. van der Goot, unpublished data.
relatively rapid phase of \([\text{Ca}^{2+}]\), change was followed by a more sustained phase.

Thus, the toxin-induced \([\text{Ca}^{2+}]\) response in myeloid cells was dose-dependent, accelerated by preactivation of the toxin, and depended on the state of differentiation of the cells. As the responses were most pronounced in HL-60 granulocytes stimulated with the mature toxin, these conditions were used to further analyze the mechanisms underlying the complex \([\text{Ca}^{2+}]\) response to aerolysin.

**Aerolysin Induces \([\text{Ca}^{2+}]\) Release from Intracellular Stores**—To investigate the source of the aerolysin-induced \([\text{Ca}^{2+}]\) elevations, we exposed HL-60 granulocytes to aerolysin in a \([\text{Ca}^{2+}]\)-free medium. Under these conditions only \([\text{Ca}^{2+}]\) release from intracellular stores can be detected, but not \([\text{Ca}^{2+}]\) influx across the plasma membrane. As shown in Fig. 4A, aerolysin (100 ng/ml) was able to induce \([\text{Ca}^{2+}]\) elevations in a \([\text{Ca}^{2+}]\)-free medium, demonstrating that the toxin triggered \([\text{Ca}^{2+}]\) release from intracellular stores. The observed \([\text{Ca}^{2+}]\) elevations had complex kinetics. An initial phase peaked and decayed after approximately 40–60 s. A prolonged phase which increased toward a plateau could be observed 1–3 min after toxin addition. The binding competent, insertion-incompetent mutant G202C/I445C did not induce \([\text{Ca}^{2+}]\) release (Fig. 4A). However, when added in excess, the mutant was able to preclude \([\text{Ca}^{2+}]\) release in response to wild type aerolysin (Fig. 4B) confirming that the two variants of the toxin have the same accepctor sites on the cell and that they are limited in number.

Many agonists induce \([\text{Ca}^{2+}]\) release from intracellular stores through phospholipase C (PLC)-mediated \([\text{Ins}(1,4,5)\text{P}_3]\) generation and \([\text{Ins}(1,4,5)\text{P}_3]\)-induced \([\text{Ca}^{2+}]\) release from intracellular stores. To test whether PLC activation is involved in the aerolysin-induced \([\text{Ca}^{2+}]\) release from intracellular stores, we have used the PLC inhibitor U73122. As shown in Fig. 4C, this compound inhibited the initial phase of the aerolysin-induced \([\text{Ca}^{2+}]\) release, however, neither the late phase of the \([\text{Ca}^{2+}]\) release, nor the \([\text{Ca}^{2+}]\) influx observed after \([\text{Ca}^{2+}]\) readdition were affected.

**Aerolysin Activates Pertussis Toxin-sensitive G-proteins**—For many granulocyte agonists, \([\text{Ca}^{2+}]\) release from intracellular stores is due to a G-protein-mediated activation of phospholipase C. In contrast to what is observed on many other cellular systems, agonist-PLC coupling in leukocytes is generally mediated by pertussis toxin-sensitive G-proteins (26). We therefore investigated the effect of pertussis toxin pretreatment on aerolysin-induced \([\text{Ca}^{2+}]\) release in the HL-60 granulocytes. As shown in Fig. 5, pertussis toxin pretreatment inhibited the initial, rapid phase of aerolysin-induced \([\text{Ca}^{2+}]\) release (Fig. 5D), but not the second, slower, phase (Fig. 5E), nor the calcium entry across the plasma membrane. These results demonstrate an activation of pertussis toxin-sensitive G-proteins through aerolysin. The results obtained with pertussis toxin (Fig. 5) and the PLC inhibitor (Fig. 4), however, also demonstrate that there is a second phase of \([\text{Ca}^{2+}]\) release which does not involve the G-protein/PLC/\([\text{Ins}(1,4,5)\text{P}_3]\) pathway.

**Aerolysin Released \([\text{Ca}^{2+}]\) from Thapsigargin and Agonist-sensitive \([\text{Ca}^{2+}]\) Stores**—A variety of intracellular organelles are able to serve as intracellular \([\text{Ca}^{2+}]\) stores, the functionally most important of which is thought to be the endoplasmatic reticulum (ER). The ER can be subdivided into agonist-sensitive and agonist-insensitive \([\text{Ca}^{2+}]\) stores. The ER \([\text{Ca}^{2+}]\) stores are, in most cases, loaded through \([\text{Ca}^{2+}]\) pumps which belong to the group of the so-called SERCa (sarcoplasmatic reticulum Ca\textsuperscript{2+}-ATPases). All sarcoplasmatic reticulum \([\text{Ca}^{2+}]\) -ATPases known to date can be inhibited by thapsigargin and are also the only known target of this drug. In addition, this compound efficiently empties ER-type \([\text{Ca}^{2+}]\) stores in many cell types. We therefore investigated the effect of depletion of ER-type \([\text{Ca}^{2+}]\) stores through thapsigargin on the aerolysin-induced \([\text{Ca}^{2+}]\) signal. As shown in Fig. 6A, thapsigargin led, as expected, to a transient increase in \([\text{[Ca}^{2+}]\)\), (Fig. 6A, see also Ref. 27). When aerolysin was added to cells after thapsigargin treatment, both phases of the aerolysin-induced \([\text{Ca}^{2+}]\) release were almost completely abolished. Thus, the source of \([\text{Ca}^{2+}]\) release by aerolysin was the endoplasmatic reticulum. Thapsigargin did, however, not inhibit channel formation by aerolysin since \([\text{Ca}^{2+}]\) entry and rapid release of intracellular \([\text{K}^{+}]\) were still observed.

To investigate whether aerolysin induces \([\text{Ca}^{2+}]\) release from agonist-sensitive stores, we stimulated cells with the receptor agonist fMet-Leu-Phe (fMLP) in a \([\text{Ca}^{2+}]\)-free medium. Under these conditions, \([\text{Ca}^{2+}]\) is released from agonist-sensitive \([\text{Ca}^{2+}]\) stores and these stores remain depleted (23). The predepletion of agonist-sensitive \([\text{Ca}^{2+}]\) stores by fMLP abolished the initial phase, but not the late phase of aerolysin-induced \([\text{Ca}^{2+}]\) release (Fig. 6, B and C). Thus, the source of the initial phase of aerolysin-induced \([\text{Ca}^{2+}]\) release were agonist-sensitive \([\text{Ca}^{2+}]\) stores, while the source of the second phase also included agonist-insensitive ER-type \([\text{Ca}^{2+}]\) stores.

**Aerolysin Induced \([\text{Ca}^{2+}]\) Influx**—As already visible from Figs. 3 and 4, the effect of aerolysin on \([\text{[Ca}^{2+}]\)\) also included a major \([\text{Ca}^{2+}]\) influx component. When \([\text{Ca}^{2+}]\) was added to the cells (in a \([\text{Ca}^{2+}]\)-free medium) after different times of incubation with aerolysin, a time-dependent increase in the \([\text{Ca}^{2+}]\) permeability was observed, as witnessed by more rapid increase in fura-2 fluorescence (Fig. 7, A and B). These observations suggest that as more aerolysin pores were formed, the kinetics of \([\text{Ca}^{2+}]\) entry were faster. We then analyzed the kinetics of entry of Mn\textsuperscript{2+}, a \([\text{Ca}^{2+}]\) surrogate commonly used to study \([\text{Ca}^{2+}]\) influx pathways. This divalent cation permeates in small quantities through various \([\text{Ca}^{2+}]\) channels; once inside the cell, Mn\textsuperscript{2+} binds with high affinity (−100-fold higher than \([\text{Ca}^{2+}]\)) to fura-2, thereby quenching the fura-2 fluorescence. As shown in Fig. 7B, the kinetics of Mn\textsuperscript{2+} and \([\text{Ca}^{2+}]\) influxes were very similar. In order to rule out that the increase in divalent cation influx kinetics were due to massive fura-2 release, we measured the amount of extracellular fura-2 as described under “Experimental Procedures.” Less than 20% fura-2 was released into the medium after treatment of HL-60 granulocytes with aerolysin (100 ng/
added as indicated. The
stores and Ca2+
B (control). (100 ng/ml), the inactive mutant G2202C/I445C (100 ng/ml), or buffer
toxin, mutant or wild type, was added.

Caerolysin (100 ng/ml).
recorded in fura-2-loaded HL-60 granulocytes incubated in Ca 2+

Given the fact that aerolysin led to a potassium efflux and
plasma membrane depolarization with kinetics similar to the ones observed for divergent cation influx, it is likely that Ca2+
enters through the pores formed by the toxin in the plasma membrane. It has indeed been previously suggested that Ca2+
channel formation in the plasma membrane is G-protein independent (Fig. 5 and not shown).

Aerolysin-induced Ca2+ Release Occurs After a Lag Time—
Fig. 8 shows, with high resolution, the kinetics of aerolysin-induced Ca2+ release. Strikingly, there was a substantial lag time between the addition of aerolysin and the onset of Ca2+
release in contrast to what is observed upon stimulation with the receptor agonist fMLP. Moreover, the lag time was concentration dependent (Fig. 8C). Thus, the marked lag time ob-
served in response to aerolysin clearly differentiated Ca2+
release in response to the toxin from Ca2+ release in response to typical receptor agonists.

Effect of Digitonin, Staphylococcal \( \alpha \)-Toxin, and Streptolysin O on Intracellular Ca2+—To further understand the mechan-
ism of G-protein activation by aerolysin, we studied Ca2+
signaling by other widely used cell permeabilizing agents such as digitonin, staphylococcal \( \alpha \)-toxin, and streptolysin O. Digi-
tonin is a plant lipid which, at low concentrations preferen-
tially binds to cholesterol and thereby leads to plasma mem-
brane permeabilization. As shown in Fig. 9A, addition of digitonin to fura-2-loaded cells suspended in a Ca2+-free me-
dium led to a drop in fluorescence intensity. Upon subsequent addition of Ca2+ to the extracellular medium, a rapid and large increase in fluorescence was observed. These results indicate that permeabilization was efficient but also that digitonin does not trigger release of Ca2+ from intracellular stores. This ob-
servation is not so surprising since digitonin has been widely
used as a permeabilizing agent in studies on the function of intracellular Ca2+ stores in large variety of cell types, including granulocytes (30). Thus, G-protein activation and Ca2+ release
from intracellular stores are not simply due to cell
dermalization.

Interestingly, however, we found that the two other pore-
forming bacterial toxins we have studied, staphylococcal \( \alpha \)-toxin and streptolysin O, both induced Ca2+ release from intracellular stores (Fig. 9B). Moreover, the Ca2+ release trig-
erged by both toxins was partly blocked by pertussis toxin (Fig.

![FIG. 3. \([\text{Ca}^{2+}]_i\) elevations in response to proaerolysin and trypsin-activated aerolysin. \([\text{Ca}^{2+}]_i\) was recorded in fura-2-
loaded HL-60 promyelocytes (A and C) and
HL-60 granulocytes (B and D) incubated in Ca2+
buffer. At times indicated by an arrow,
cells were treated with either proaerolysin (A and B) or trypsin-activated aerolysin (C and D). All experiments were performed with the wild type toxin, except for the indicated curve in D, where the cells were treated with the trypsin-activated G202C/I445C inactive mutant.

![FIG. 4. Aerolysin-dependent Ca2+ release from intracellular
stores and Ca2+ influx across the plasma membrane. \([\text{Ca}^{2+}]_i\) was recorded in fura-2-loaded HL-60 granulocytes incubated in Ca2+-free buffer. However, near the end of each experiment, 1 mM Ca2+ was added as indicated. The unlabeled arrows indicate the time at which the toxin, mutant or wild type, was added. A, addition of wild type aerolysin (100 ng/ml), the inactive mutant G2202C/I445C (100 ng/ml), or buffer (control). B, addition of an excess of inactive mutant G2202C/I445C (5 

mu g/ml) or buffer (no mutant) was followed by addition of wild type aerolysin (100 ng/ml). C, addition of wild type aerolysin (100 ng/ml) to cells that had been preincubated with or without the phospholipase C inhibitor U73122 (2 

mu M, 5 min).

![Added text]treated cells, divalent cations can enter the cell through the pores formed by the toxin in the plasma membrane. As sus-
ppected, neither the aerolysin-induced Ni2+ or Ca2+ influx was blocked by pertussis toxin, indicating that channel formation in the plasma membrane is G-protein independent (Fig. 5 and not shown).

Aeolysin is able to diffuse through the aerolysin channel (28). However,
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release in contrast to what is observed upon stimulation with the receptor agonist fMLP. Moreover, the lag time was concent-
ration dependent (Fig. 8C). Thus, the marked lag time ob-
served in response to aerolysin clearly differentiated Ca2+
release in response to the toxin from Ca2+ release in response to typical receptor agonists.

Effect of Digitonin, Staphylococcal \( \alpha \)-Toxin, and Streptolysin O on Intracellular Ca2+—To further understand the mechan-
ism of G-protein activation by aerolysin, we studied Ca2+
signaling by other widely used cell permeabilizing agents such as digitonin, staphylococcal \( \alpha \)-toxin, and streptolysin O. Digi-
tonin is a plant lipid which, at low concentrations preferen-
tially binds to cholesterol and thereby leads to plasma mem-
brane permeabilization. As shown in Fig. 9A, addition of digitonin to fura-2-loaded cells suspended in a Ca2+-free me-
dium led to a drop in fluorescence intensity. Upon subsequent addition of Ca2+ to the extracellular medium, a rapid and large increase in fluorescence was observed. These results indicate that permeabilization was efficient but also that digitonin does not trigger release of Ca2+ from intracellular stores. This ob-
servation is not so surprising since digitonin has been widely
used as a permeabilizing agent in studies on the function of intracellular Ca2+ stores in large variety of cell types, including granulocytes (30). Thus, G-protein activation and Ca2+ release
from intracellular stores are not simply due to cell
dermalization.

Interestingly, however, we found that the two other pore-
forming bacterial toxins we have studied, staphylococcal \( \alpha \)-toxin and streptolysin O, both induced Ca2+ release from intracellular stores (Fig. 9B). Moreover, the Ca2+ release trig-
erged by both toxins was partly blocked by pertussis toxin (Fig.
Aerolysin Triggers Ca\textsuperscript{2+} Release from Intracellular Stores

**Fig. 5.** Aerolysin-induced Ca\textsuperscript{2+} release from intracellular stores involves pertussis toxin-sensitive G-proteins. HL-60 granulocytes were pretreated or not with pertussis toxin (500 ng/ml) for 1 h at 37 °C, and subsequently loaded with fura-2 as described under “Experimental Procedures.” Cells, suspended in Ca\textsuperscript{2+}-free buffer, were then exposed to 10 (A), 100 (B), or 1000 (C) ng/ml of aerolysin at the times indicated by an arrow. Near the end of each experiment, Ca\textsuperscript{2+} (1 mM) followed by the calcium ionophore ionomycin (2 \mu M) were added as indicated. D, percentage of increase in fura-2 fluorescence intensity at the first peak after toxin addition (i.e. peak fluorescence minus basal fluorescence) was plotted as function of aerolysin concentration for pertussis toxin treated and control cells. E, percentage of increase in fura-2 fluorescence intensity at 3 min after toxin addition (i.e. 3-min fluorescence minus basal fluorescence) was plotted as a function of the time elapse between the addition of aerolysin (100 ng/ml, second arrow) followed by the addition of thapsigargin (100 nM), or the equivalent volume of Me\textsubscript{2}SO (first arrow). F, percentage of increase in fura-2 fluorescence intensity after 1 and 3 min after aerolysin addition in control (Me\textsubscript{2}SO treated), thapsigargin, and fMLP-treated cells.

**Fig. 6.** Aerolysin leads to the release of Ca\textsuperscript{2+} from thapsigargin and agonist-sensitive stores. [Ca\textsuperscript{2+}], was recorded in fura-2-loaded HL-60 granulocytes incubated in Ca\textsuperscript{2+}-free buffer. A, addition of the Ca\textsuperscript{2+}-ATPase inhibitor thapsigargin (100 nM) or the equivalent volume of Me\textsubscript{2}SO (first arrow) followed by the addition of aerolysin (100 ng/ml, second arrow). B, addition of the receptor agonist fMLP (1 \mu M) or the equivalent volume of Me\textsubscript{2}SO (first arrow) followed by the addition of aerolysin (100 ng/ml, second arrow). C, percentage of increase in fura-2 fluorescence intensity after 1 and 3 min after aerolysin addition in control (Me\textsubscript{2}SO treated), thapsigargin, and fMLP-treated cells.

**Fig. 7.** Aerolysin-induced influx of divalent cations. A, [Ca\textsuperscript{2+}], was recorded in fura-2-loaded HL-60 granulocytes incubated in Ca\textsuperscript{2+}-free buffer. The fluorescence was monitored using an excitation wavelength of 340 nm. After different times, Ca\textsuperscript{2+} was added to the extracellular medium (1 mM). B, the percentage of fluorescence increase that occurred within 10 s after addition of Ca\textsuperscript{2+} to the extracellular medium was plotted as a function of the time elapse between the addition of aerolysin and the addition of Ca\textsuperscript{2+}. Similarly the percentage of fluorescence quenching that occurred within 1 min after addition of Mn\textsuperscript{2+} was plotted as a function of the time elapse between the addition of aerolysin and the addition of Mn\textsuperscript{2+} (25 \mu M). C, [Ca\textsuperscript{2+}], was recorded in fura-2-loaded HL-60 granulocytes incubated in Ca\textsuperscript{2+}-free buffer. The fluorescence was monitored using an excitation wavelength \textlambda\textsubscript{ex} of 360 nm, which corresponds to the isosbestic point. The first arrow indicates the addition of aerolysin (100 ng/ml) and the second the addition of Ni\textsuperscript{2+} (100 \mu M). D, [Ca\textsuperscript{2+}], was recorded in fura-2-loaded HL-60 granulocytes incubated in Ca\textsuperscript{2+}-free buffer (\textlambda\textsubscript{ex} = 360 nm). Thapsigargin (100 nM) was added or not at the first arrow and Ni\textsuperscript{2+} (100 \mu M) at the second arrow.

9C) as previously observed for aerolysin. The observation that \alpha-toxin triggers Ca\textsuperscript{2+} release is difficult to reconcile with previous reports suggesting that staphylococcal \alpha-toxin exclusively triggers Ca\textsuperscript{2+} influx across the plasma membrane (31–33). Note, however, that Suttrop and Habben (31) also did not detect a [Ca\textsuperscript{2+}], increase in response to the Ca\textsuperscript{2+} ionophore ionomycin, suggesting that the experimental conditions were not optimized with respect to detection of Ca\textsuperscript{2+} release.

**Pertussis Toxin-sensitive Chemotaxis in Response to Aerolysin, \alpha-Toxin, and Streptolysin O—**To investigate whether toxin-dependent G-protein activation activates cell functions, we investigated the effect of aerolysin, \alpha-toxin, and streptolysin O on chemotaxis of freshly prepared human blood granulocytes (Fig. 10). Under control conditions, less than 0.5% of the cells that had been added in the cell reservoir of the chemotaxis chamber were recovered in the target chamber. In contrast, when aerolysin, \alpha-toxin, or streptolysin O were added to the target chamber, significant chemotactic activity was observed. The chemotactic activity observed in response to the toxins was of a similar magnitude as observed with classical chemottractants, such as fMLP or platelet-activating factor (see also legend of Fig. 10). Importantly, chemotaxis in response to the toxins could be completely blocked by preincubation of cells with pertussis toxin, demonstrating that the activation of G-proteins by the toxins was essential for the induction of chemotaxis.
FIG. 8. Kinetics of early changes in fura-2 fluorescence upon aerolysin or fMLP treatment. HL-60 granulocytes were incubated in Ca\(^{2+}\)-free buffer. The changes in fura-2 fluorescence were monitored upon addition of digitoxin (A, 12.5 μM), staphylococcal α-toxin (B, 10 μg/ml), and streptolysin O (C, 50 ng/ml). Near the end of each experiment, Ca\(^{2+}\) (1 mM) was added as indicated.

FIG. 9. Effect of membrane permeabilizing agents on the Ca\(^{2+}\) fluxes. HL-60 granulocytes were incubated in Ca\(^{2+}\)-free buffer. The first phase of aerolysin-induced Ca\(^{2+}\) release was more sustained, but of a relatively low amplitude. It did not occur through a G-protein and PLC pathway. This phase was completely abolished by pretreatment of cells with thapsigargin, but only partially by pretreatment with fMLP. Thus, the source of the second release phase have as yet to be determined. Generation of a yet unknown signal at the plasma membrane might occur. Alternatively, a direct action of aerolysin on the endoplasmic reticulum might be considered.

Aerolysin-induced Ca\(^{2+}\) Release from Intracellular Stores—A pore-forming toxin is expected to allow ion fluxes across the plasma membrane. In accordance with this prediction, our results demonstrate that aerolysin increases the plasma membrane permeability for monovalent and divalent cations in granulocytes. However, aerolysin-induced Ca\(^{2+}\) elevations were more complex than anticipated. Experiments performed in the absence of extracellular Ca\(^{2+}\) revealed that an early event triggered by aerolysin was the release of Ca\(^{2+}\) from intracellular stores. The Ca\(^{2+}\) release occurred in two phases. The first phase of aerolysin-induced Ca\(^{2+}\) release was rapid and transient. It could be inhibited by pertussis toxin as well as the phospholipase C inhibitor U73122, indicating that it involves the activation of a G-protein and PLC. This phase was also abolished when agonist-sensitive Ca\(^{2+}\) stores were predepleted by exposure of cells to the Ins(1,4,5)P\(_3\)-generating receptor agonist fMLP. Thus, the early phase of aerolysin-induced Ca\(^{2+}\) release were most likely Ins(1,4,5)P\(_3\)-sensitive endoplasmic reticulum Ca\(^{2+}\) stores. The second phase of aerolysin-induced Ca\(^{2+}\) release was more sustained, but of a relatively low amplitude. It did not occur through a G-protein phospholipase C pathway. This phase was completely abolished by pretreatment of cells with thapsigargin, but only partially by pretreatment with fMLP. Thus, the source of the second phase of Ca\(^{2+}\) release most likely comprised not only Ins(1,4,5)P\(_3\)-sensitive, but also Ins(1,4,5)P\(_3\)-insensitive endoplasmic reticulum Ca\(^{2+}\) stores. The mechanisms underlying the second release phase have as yet to be determined. Generation of a yet unknown signal at the plasma membrane might occur. Alternatively, a direct action of aerolysin on the endoplasmic reticulum might be considered.

Aerolysin-induced G-Protein Activation—Our results clearly suggest that aerolysin activates pertussis toxin-sensitive G-proteins in granulocytes. This possibility has also been suggested by previous reports on the block of aerolysin-induced granulocyte chemotaxis by pertussis toxin (34). A most
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with the proaerolysin producing strain and the purified proaerolysin mutant G202C/1445C, Beatrix Vecsey-Semjen for giving us a-toxin, and M. Kehoe for giving us purified streptolysin O. We are very grateful to Nathalie Madore for going through all the trouble of providing us with an anti-Thy-1 antibody and are thankful to Roger Morris for giving us the antibody. We thank Eduardo Martinez for human T lymphocytes and Hai-Tao He for mouse T lymphocytes.

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straightforward explanation for the activation of G-proteins by aerolysin would be the following: the aerolysin receptor on granulocytes is a G-protein-coupled receptor and binding of aerolysin to this protein therefore induces G-protein activation. However, for several reasons, we think that this explanation is unlikely. First, the insertion-incompetent G202C/1445C aerolysin mutant efficiently binds to the same cell surface receptors as the wild type toxin, as evidenced by the almost complete block of aerolysin-induced Ca\(^{2+}\) release by the preincubation with the mutant. However, the mutant did not induce Ca\(^{2+}\) release. Second, the kinetics of increase in [Ca\(^{2+}\)]\(_{c}\) were slower than those triggered by the active form of the toxin, suggesting again that pore formation and not binding is crucial for G-protein activation. Third, all proaerolysin receptors identified so far are GPI-anchored proteins and not transmembrane proteins. Finally, the existence of a lag time between the addition of the toxin and the onset of Ca\(^{2+}\) release argues against a direct binding to a G-coupled receptor. Indeed no lag is observed upon addition of receptor agonists such as IMLP. Additional evidence that aerolysin-induced G-protein activation is a consequence of pore formation, rather than of receptor binding comes from the observation that G-protein activation is a common theme observed in response to pore formation by a variety of proteins and peptides.
Aerolysin Induces G-protein Activation and Ca$^{2+}$ Release from Intracellular Stores in Human Granulocytes
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