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Sensitivity of Polarized Epithelial Cells to the Pore-Forming Toxin Aerolysin

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Aerolysin is one of the major virulence factors produced by *Aeromonas hydrophila*, a human pathogen that produces deep wound infection and gastroenteritis. The toxin interacts with target mammalian cells by binding to the glycan core of glycosylphosphatidyl inositol (GPI)-anchored proteins and subsequently forms a pore in the plasma membrane. Since epithelial cells of the intestine are the primary targets of aerolysin, we investigated its effect on three types of polarized epithelial cells: Caco-2 cells, derived from human intestine; MDCK cells, a well-characterized cell line in terms of protein targeting; and FRT cells, an unusual cell line in that it targets its GPI-anchored proteins to the basolateral plasma membrane in contrast to other epithelial cells, which target them almost exclusively to the apical surface. Surprisingly, we found that all three cell types were sensitive to the toxin from both the apical and the basolateral sides. Apical sensitivity was always higher, even for FRT cells. In contrast, FRT cells were more sensitive from the basolateral than from the apical side to the related toxin *Clostridium septicum* alpha-toxin, which also binds to GPI-anchored proteins but lacks the lectin binding domain found in aerolysin. These observations are consistent with the notion that a shuttling mechanism involving low-affinity interactions with surface sugars allows aerolysin to gradually move toward the membrane surface, where it can finally encounter the glycan cores of GPI-anchored proteins.

The pore-forming toxin aerolysin is produced by the human enteropathogen *Aeromonas hydrophila* (for a review, see reference 18). The toxin is secreted as an inactive precursor, proaerolysin (25), which diffuses toward its target cell, to which it binds via specific receptors. A variety of receptors have been identified on different cell types, and all have the common property of being glycosylphosphatidyl inositol (GPI) anchored (2, 16, 35). Also, screening for aerolysin resistance leads to cells that are deficient in GPI biosynthesis (3). The identified proaerolysin binding proteins include Thy-1 (35), CD14, and semaphorin 7 (20). Since none of these proteins show any sequence homology, it appears that aerolysin recognizes the glycan cores of the GPI anchors (16). Binding to protein-free GPI, however, does not occur, suggesting that the attached protein also plays a role in binding (5). This was recently confirmed by the observation that high-affinity binding of aerolysin to its receptor requires not only the glycan core of the GPI anchor but also an N-glycan on the attached protein. More specifically, this study showed that aerolysin binds only to mature N-glycan, the minimal N-glycan structure being Gn-M_3-Gn_2-N (Gn, *N*-acetylglucosamine; M, mannose; N, asparagine), which is obtained after trimming by Golgi mannosidase II (24).

After binding to the cell surface, proaerolysin is processed into aerolysin by the endoprotease furin or other members of the proprotein convertase family (1). Alternatively, proaerolysin can be cut by digestives enzymes, such as trypsin and chymotrypsin, prior to receptor binding. Once processed, the toxin must oligomerize into a ring-like heptameric structure (33, 38) that inserts into membranes and forms channels. This step is favored by the association of receptor bound toxin with specialized cholesterol-rich microdomains, or lipid rafts (19, 23, 26), of the plasma membrane, which leads to a local increase in the toxin concentration (4). Finally, channel formation leads to permeabilization of the plasma membrane to small ions (1, 2), activation of G-proteins (29), and apoptosis (34) in certain cell types.

Although *A. hydrophila* can produce deep wound infection and septicemia, mainly in immunocompromised hosts, it is generally associated with acute gastroenteritis in both children and adults, and strong evidence implicates aerolysin as an important virulence factor produced by the bacterium (14, 17, 27, 28). Therefore, the main targets of aerolysin are likely to be the epithelial cells of the intestine. However, little is known about the interaction of aerolysin with epithelial cells. All of the above-mentioned studies were performed on fibroblast-like cells (1, 2, 4), lymphocytes (34, 35), granulocytes (29), or erythrocytes (13). Here, we have studied the interaction of aerolysin with polarized epithelial cells. In addition to being potential physiological targets for the toxin, polarized epithelial cells are also interesting in the context of aerolysin because of the documented asymmetric distribution of GPI-anchored proteins (32). GPI-anchored proteins predominantly, if not exclusively, distribute to apical plasma membrane, as shown for

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MDCK cells and Caco-2 cells (10, 30, 32, 37). Apical targeting appears to be ensured both by the GPI anchor (31) and by the N-linked sugars on the protein (9). A notable exception among epithelial cells is the Fisher rat thyroid (FRT) cell line, which preferentially targets its GPI-anchored proteins to the basolateral membrane (40).

In the present work, we have analyzed the effects of aerolysin on the three above-mentioned polarized epithelial cell lines: Caco-2 cells, because they are derived from human intestine and therefore constitute an attractive model to study the effect of aerolysin; MDCK cells, because they are the best-characterized polarized cell line in terms of protein targeting; and finally FRT cells, since they show an altered distribution of GPI-anchored proteins.

MATERIALS AND METHODS

Cell culture and toxin purification. MDCK and Caco-2 cells were grown and maintained in Dulbecco's modified Eagle's medium (1:1; Sigma Chemical Co., St. Louis, Mo.) complemented with 10% fetal calf serum–2 mM L-glutamine–4.5 g of glucose/liter under standard tissue culture conditions. FRT cells were grown in Coon's modified F12 medium complemented with 5% fetal calf serum–2 mM L-glutamine–4.5 g of glucose/liter under standard tissue culture conditions (40). All experiments on polarized cells were performed on COSTAR filter-grown cells. MDCK and FRT cells were grown on the filters for 4 days, and Caco-2 cells were grown for 12 days.

Proaerolysin was purified as described previously (12). The alpha-toxin was purified from the culture supernatant of *Clostridium septicum* strain KZ1003 (a gift from Shinichi Nakamura, Kanazawa University, Kanazawa, Japan). The toxin was precipitated from the supernatant of an 18-h culture in brain heart infusion broth (Difco, Detroit, Mich.) by 60% saturated ammonium sulfate; dissolved in 10 mM sodium phosphate, pH 7.0; and chromatographed on a cation exchanger, SP-Toyopearl 650 M. The purified preparation of the toxin gave a single protein band at a position corresponding to a molecular weight of 48,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The alpha-toxin was conjugated with *N*-hydroxysuccinimide–biotin (Pierce) according to the manufacturer's instructions.

TER. The transepithelial electrical resistance (TER) was determined by applying an AC square-wave current of ± 20 μA at 12.5 Hz with a silver electrode and measuring the voltage deflection elicited with a silver-silver chloride electrode using an EVOM (World Precision Instruments) as described previously (6). TER values were obtained by subtracting the contributions of the filter and bath in solution. TER changes upon toxin treatment were expressed as percentages of the TER at time zero. All experiments were performed at 37°C. For these experiments, cells were grown on filters with a 24-mm diameter. During the experiments, 1.5 ml of incubation medium (IM) was added to the apical compartment and 3 ml was added to the basolateral compartment. The cells were incubated for 30 min in IM at 37°C prior to the first TER measurement.

Potassium efflux measurements. Confluent monolayers grown on 24-mm-diameter filters were washed once and incubated in IM containing Glasgow minimal essential medium buffered with 10 mM HEPES, pH 7.4, for 30 min at 37°C in the absence of proaerolysin. The monolayers were then further incubated at 37°C with proaerolysin in IM for various times. The cells were subsequently washed with ice-cold potassium-free choline medium, pH 7.4, containing 129 mM choline-Cl, 0.8 mM $MgCl_2$, 1.5 mM $CaCl_2$, 5 mM citric acid, 5.6 mM glucose, 10 mM NH_4Cl, and 5 mM H_3PO_4 and solubilized with 0.5% Triton X-100 in the same buffer for 20 min at 4°C. The potassium contents of the cell lysates were determined by flame emission photometry using a Philips PYE UNICAM SP9 atomic adsorption spectrophotometer.

PI-PLC treatment. Cells grown to confluence on 24-mm-diameter filters were incubated from either the apical side (0.5 ml) or the basolateral side (1 ml) with 6 U of phosphatidylinositol-specific phospholipase C (PI-PLC)/ml in IM for 2 h at 37°C in the presence of 10 μg of cycloheximide/ml. The effect of proaerolysin on the intracellular potassium content was then measured as described above.

Triton X-100 insolubility and preparation of DRMs. The cells were treated with ^{125}I-proaerolysin, scraped from the filter, harvested by centrifugation, and resuspended in 0.5 ml of cold buffer containing 25 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100, as well as Complete, a cocktail of protease inhibitors (Boehringer Mannheim). The membranes were solubilized by rotary shaking at 4°C for 30 min. The detergent extraction was performed as previously described (2, 11, 21), and detergent resistant membranes (DRMs) were purified on a sucrose gradient as follows. The sample was adjusted to 40.3% sucrose in 10 mM Tris-HCl, pH 7.4, in an SW40 Beckman tube, overlaid with 8.5 ml of 35% sucrose, topped up with 15% sucrose, and centrifuged for 18 h at 35,000 rpm in a Beckman Sw40 rotor at 4°C. Twelve 1-ml fractions were collected and counted in a gamma spectrometer.

Swainsonine treatment and detection of complex N-glycans. MDCK and FRT cells grown on filters were treated with swainsonine at 10 μg/ml for 4 days from both sides or left untreated. The cells were incubated from the apical or basolateral side with 10 μg of fluorescein isothiocyanate–phytohemagglutinin-P (PHA-P)/ml for 30 min at 4°C, scraped from the filters, and mechanically lysed. The fluorescences of the cell extracts were measured with a PTI spectrofluorimeter using an excitation wavelength of 490 nm and an emission wavelength of 520 nm.

RESULTS

Effect of proaerolysin on the TER of polarized epithelial cells. To test whether Caco-2, MDCK, and FRT cells are sensitive to proaerolysin, we measured the effect of the toxin on the TER of cell monolayers. The toxin was added to filter-grown cells either to the apical compartment alone or to the basolateral compartment alone. Proaerolysin led to a drop in TER in all three cell types (Fig. 1). A lag time was observed for Caco-2 and FRT cells but not for MDCK cells, which had a 10-fold-lower initial TER, suggesting that tight monolayers are more resistant to aerolysin. Surprisingly, Caco-2 and MDCK cells were sensitive not only to apical exposure to aerolysin, as expected from the published distribution of GPI-anchored proteins, but also to basolateral exposure to the toxin (Fig. 1A and B). The kinetics of a drop in the TER of MDCK cells were identical whether the toxin was added apically or basolaterally; however, for Caco-2 cells, the kinetics were slower when the toxin was added basolaterally (Fig. 1A). Surprisingly also, FRT cells were sensitive not only to basolateral toxin exposure but also to apical exposure, which led to an even faster drop in the TER (Fig. 1C).

Effect of proaerolysin on the intracellular potassium contents of polarized epithelial cells. The decrease in TER is most likely an indirect consequence of pore formation by aerolysin. A number of non-mutually exclusive aerolysin-induced events could explain this drop in TER. In particular, the death of certain cells, leading to a breach in the cell monolayer, most likely occurs (2). Also, tight junctions could disassemble due to an increase in intracellular calcium (15) coming through the aerolysin channel (29). Finally, if channel formation by aerolysin indeed occurs, it will remove the resistance of the apical or basolateral membrane (since aerolysin channels are nonselective [39]), thereby contributing to the decrease in TER.

To get a more direct estimate of the pore-forming activity of aerolysin on polarized cells, we measured the change in intracellular potassium. If indeed aerolysin forms a channel in the apical or basolateral plasma membrane, this will lead to potassium efflux across the membrane (1, 2). Therefore, measuring intracellular potassium provides information on a very early event in the mode of action of the toxin. Note that in these experiments ion flux across a single membrane (the plasma membrane domain that is observed) is measured in contrast to the experiments shown in Fig. 1, where the electrical resistance is measured across an entire cell monolayer (transcellular plus paracellular fluxes).

Once more, proaerolysin was added either apically or basolaterally to filter-grown cells. As can be seen in Fig. 2, potas-

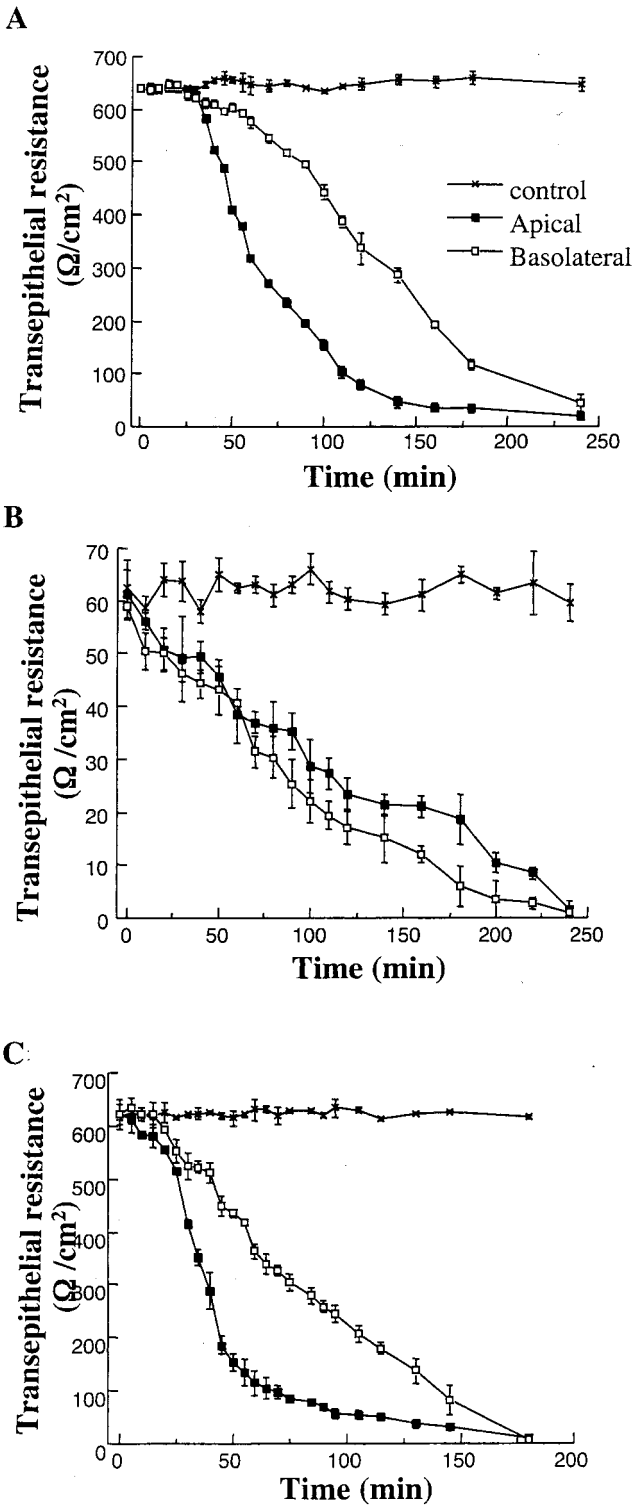

FIG. 1. Proaerolysin leads to a drop in TER. Filter-grown Caco-2 (A), MDCK (B), and FRT (C) cells were incubated with proaerolysin either from the apical side or the basolateral side of the monolayer. The TER was measured as a function of time (37°C). MDCK and FRT cells were treated with 0.38 nM toxin, and Caco-2 cells were treated with 0.95 nM toxin. The error bars indicate standard deviations.

sium efflux could be observed for all three cell types regardless of whether the toxin was added to the basolateral side or to the apical side. No lag times were observed, due to the fact that heptamer and channel formation occurs rapidly at the cell surface at the concentrations used in these experiments (4).

Interestingly, the kinetics of potassium efflux were always slower upon basolateral treatment, even for FRT cells. Similar differences in kinetics between apically and basolaterally added toxins were observed when cells were treated with trypsin-nicked aerolysin, indicating that the observed differences were not due to the potentially polarized distribution of the protoxin-processing enzymes, such as furin (not shown).

We next investigated whether the kinetics of potassium efflux correlated with the amount of toxin bound to the apical versus the basolateral membrane. Therefore, the binding of radiolabeled proaerolysin was quantified. A typical binding experiment is shown in Fig. 3A. Not surprisingly, the degree of binding varied among cells types. Importantly, for all cell types, the level of apical binding was always three to four times higher than that of basolateral binding (Fig. 3B). The systematically lower level of basolateral binding is not due to an accessibility problem in our filter setup, since we found that binding of the protective antigen of *Bacillus anthracis* was far more pronounced on the basolateral side than on the apical side (not shown), in agreement with the literature (8), as was that of *C. septicum* alpha-toxin on FRT cells (see Fig. 6B, inset).

Potassium efflux is inhibited by PI-PLC treatment on both the apical and the basolateral sides. The above observations indicate that aerolysin can form channels in both the apical and the basolateral membranes of polarized epithelial cells. The fact that the present experiments were performed at very low toxin concentrations, in the 0.4- to 1-nM range, suggests that the interaction of the toxin with the plasma membrane was specific and receptor mediated. This was confirmed by the observation that binding of radiolabeled aerolysin could be inhibited by an excess of cold toxin (not shown). Since all proaerolysin receptors so far identified are GPI anchored, we investigated whether treatment of polarized cells, from either the apical or the basolateral side, with PI-PLC would affect channel formation by aerolysin. PI-PLC treatment led to a dramatic reduction in potassium efflux whether proaerolysin was added apically or basolaterally (Fig. 4). The residual aerolysin activity after PI-PLC treatment could be due to alternative, non-GPI-anchored aerolysin receptors. More likely, however, the removal of GPI-anchored proteins was not complete due to inefficiency of the enzyme or insufficient accessibility. Moreover, there is at present no reason to invoke any high-affinity non GPI-anchored aerolysin receptors. Indeed, when we screened for aerolysin-resistant mutant CHO cells, all appeared to be deficient in GPI biosynthesis (3). In such screens, cells with reduced aerolysin sensitivity were also recently found (24). These cells were deficient in *N*-acetylglucosamine transferase I, an enzyme involved in the maturation of N-glycans (24). Importantly, this study shows that for domain 1 of aerolysin to bind to complex N-glycans, the sugars must be attached to a GPI-anchored protein (24).

These observations show that proaerolysin binds to GPI-anchored proteins present on the apical and the basolateral sides of all three polarized cell lines.

Proaerolysin is enriched in detergent-insoluble membranes

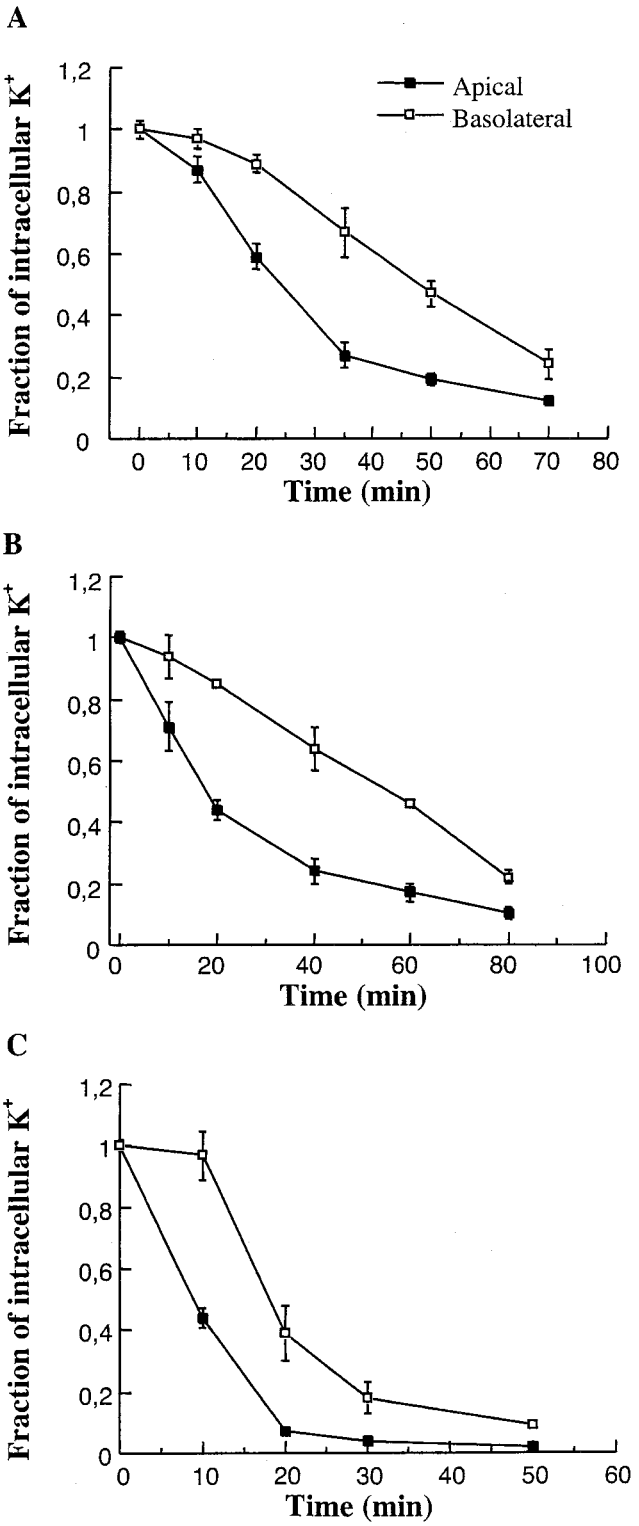

FIG. 2. Channel formation by aerolysin in the apical and basolateral membranes of polarized epithelial cells. Filter-grown Caco-2 (A), MDCK (B), and FRT (C) cells were incubated with proaerolysin from either the apical side or the basolateral side of the monolayer. The intracellular potassium of the cells was measured as a function of time (37°C) and was expressed as a fraction of the initial potassium content. MDCK and FRT cells were treated with 0.38 nM toxin, and Caco-2 cells were treated with 0.95 nM toxin. The error bars indicate standard deviations.

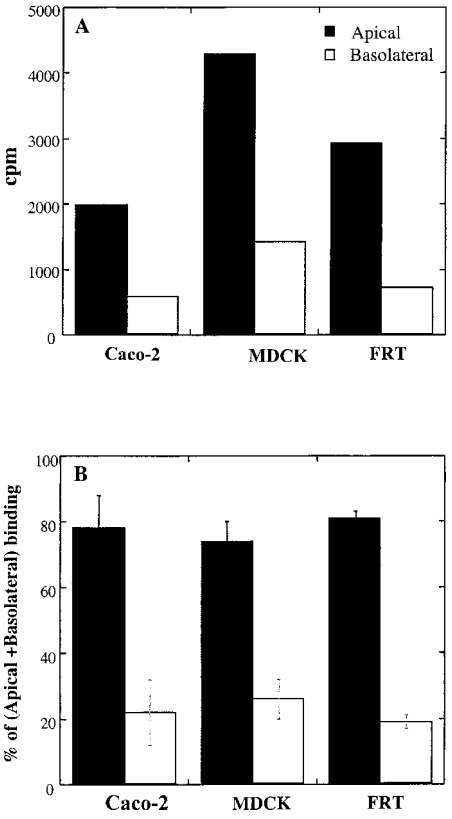

FIG. 3. Binding of aerolysin to the apical and basolateral domains of polarized epithelial cells. Filter-grown Caco-2, MDCK, and FRT cells were incubated with ^{125}I-proaerolysin from either the apical side or the basolateral side of the monolayer for 1 h at 4°C. MDCK and FRT cells were treated with 0.38 nM toxin, and Caco-2 cells were treated with 0.95 nM toxin. The cells were washed and scraped from the filter, and the total bound toxin was measured. (A) Typical experiment illustrating the greater binding of aerolysin to the apical side of all three cell types. (B) Results are expressed as percentages of total apical plus total basolateral counts. The error bars represent standard deviations ($n = 3$).

of both plasma membrane domains. In polarized cells, GPI-anchored proteins have been shown to associate with specialized microdomains of the plasma membrane called lipid rafts, which are highly enriched in cholesterol and glycosphingolipids (11). These rafts have the biochemical property of being resistant to solubilization in certain nonionic detergents at 4°C. It has been shown that the ability of the receptors to associate with lipid rafts promotes channel formation by aerolysin in fibroblasts by increasing the rate of oligomer formation (4).

To extend these observations to polarized cells, we measured the association of the toxin with DRMs on both plasma membrane domains. Proaerolysin was bound to either the apical or the basolateral side of the polarized cells at 4°C. The cells were then solubilized in Triton X-100 at 4°C, and the DRMs were purified by sucrose density centrifugation.

As can be seen in Fig. 5, 50 to 65% of the total cell-bound proaerolysin was associated with DRMs, which contain <10% of the total cellular protein (4), suggesting that proaerolysin was highly enriched in lipid rafts. The amount of toxin found associated with basolateral DRMs was smaller in FRT cells

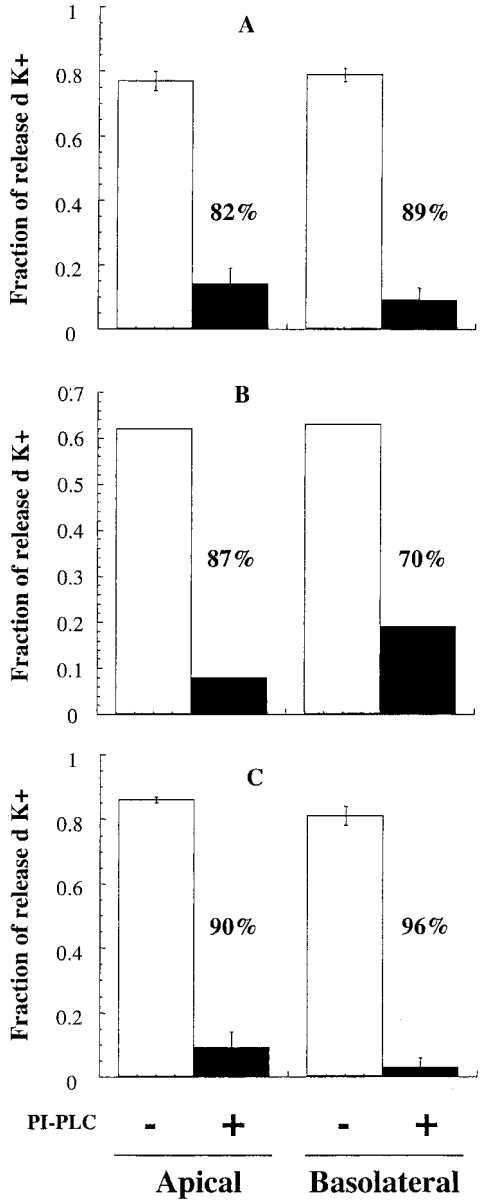

FIG. 4. PI-PLC treatment inhibits proaerolysin-induced potassium efflux from both the apical and basolateral plasma membranes of polarized epithelial cells. Filter-grown Caco-2 (A), MDCK (B), and FRT (C) cells were treated with 6 U of PI-PLC/ml for 2 h at 37°C from either the apical side or the basolateral side of the monolayer. The cells were then incubated with proaerolysin from either the apical side or the basolateral side (37°C). MDCK and FRT cells were treated with 0.38 nM aerolysin, and Caco-2 cells were treated with 0.95 nM aerolysin. The intracellular potassium of the cells was measured after 40 min for apically treated MDCK cells, after 60 min for basolaterally treated MDCK cells, after 35 min for apically treated Caco-2 cells, after 60 min for basolaterally treated Caco-2 cells, after 30 min for apically treated FRT cells, and after 50 min for basolaterally treated FRT cells. The error bars indicate standard deviations.

than in MDCK cells. This is in agreement with the observation that in FRT cells, basolateral GPI-anchored proteins are Triton X-100 soluble (42), in contrast to what was observed in MDCK cells (a GPI-anchored version of the rat growth hormone receptor, which is not glycosylated, has a nonpolarized distribution and in both membranes was found to be Triton X-100 insoluble).

Effect of *C. septicum* alpha-toxin on polarized epithelial cells. We next investigated the effect of *C. septicum* alpha-toxin on polarized epithelial cells in comparison to that of aerolysin. This toxin is homologous to aerolysin but lacks an amino-terminal domain that has lectin properties. As shown in Fig. 6, MDCK cells exhibited the same sensitivity to alpha-toxin on both plasma membrane domains, whereas FRT cells were more sensitive to alpha-toxin when exposed from the basolateral side than when exposed from the apical side, in contrast to what was observed for aerolysin. We next investigated whether the higher basolateral sensitivity of FRT cells correlated with a higher level of binding of alpha-toxin. As shown in the inset in Fig. 6B, more alpha-toxin was bound to the basolateral than to the apical side of FRT cells, in contrast to what is observed for alpha-toxin on MDCK cells and in contrast to what was observed for aerolysin on FRT cells (Fig. 3).

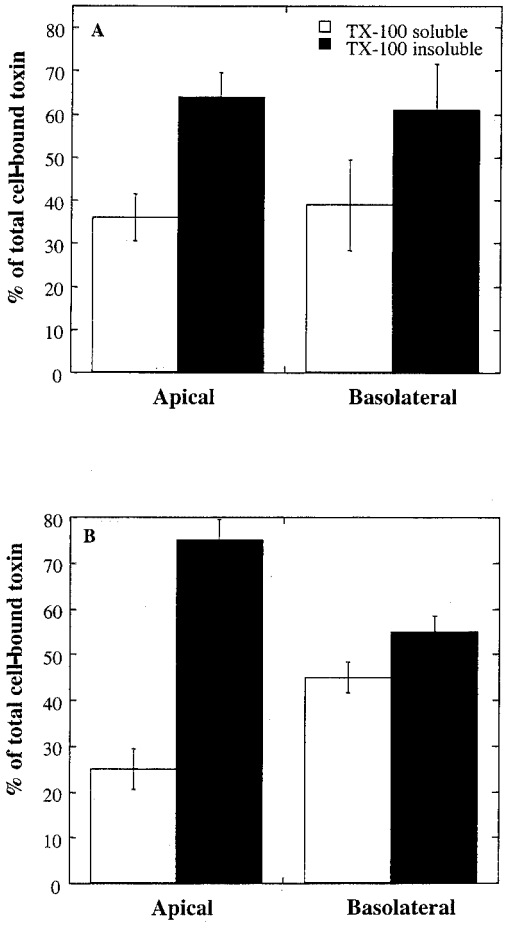

FIG. 5. Association of proaerolysin with DRMs. MDCK (A) and FRT (B) cells were treated with 0.38 nM ^{125}I-proaerolysin from either the apical side or the basolateral side of the monolayer for 1 h at 4°C. The cells were then scraped from the filter, and DRMs were prepared by flotation on sucrose gradients. The counts in the low-buoyancy fractions (corresponding to DRMs) were measured, as well as those at the bottom of the gradient, corresponding to the detergent-soluble material. The error bars indicate standard deviations. TX-100, Triton X-100.

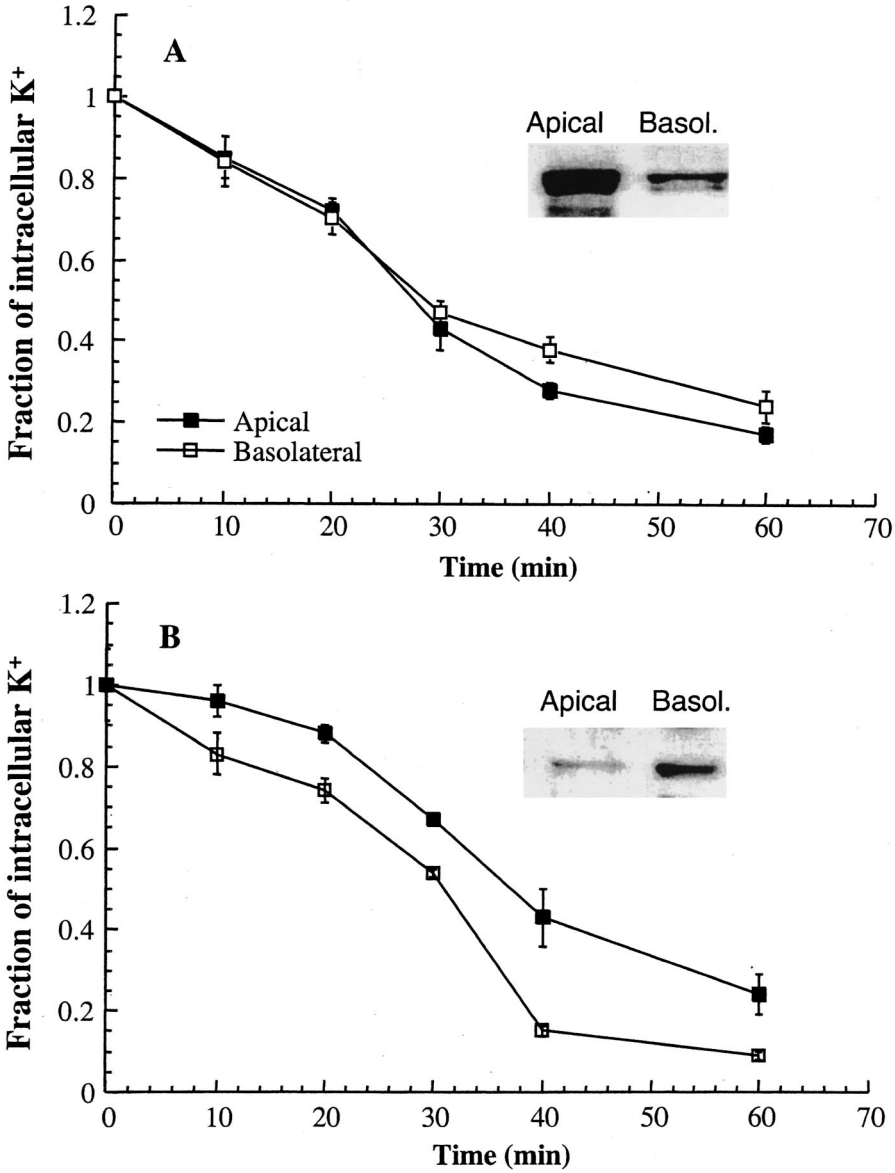

FIG. 6. Channel formation by *C. septicum* alpha-toxin in the apical and basolateral membranes of polarized epithelial cells. Filter-grown MDCK (A) and FRT (B) cells were incubated with alpha-toxin (0.43 nM) from either the apical side or the basolateral (Basol.) side of the monolayer. The intracellular potassium of the cells was measured as a function of time (37°C) and was expressed as a fraction of the initial potassium content. (Insets) MDCK (A) and FRT (B) cells grown on filters were treated with biotinylated alpha-toxin (2.1 nM) from either the apical side or the basolateral side of the monolayer. Cell extracts (20 μg) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by blotting onto a nitrocellulose membrane on which biotin–alpha-toxin was revealed using streptavidin horseradish peroxidase (Sigma). The error bars indicate standard deviations.

N-glycans are more abundant on the apical than on the basolateral side of polarized epithelial cells. Although it is well known that a thick glycocalyx is present on the apical surfaces of epithelia and that apical proteins can be highly glycosylated, we wished to confirm that on MDCK and FRT cells in culture, N-glycans are indeed more abundant on the apical plasma membrane domain. The presence of N-glycans was monitored by measuring the binding of fluorescently labeled PHA-P, a lectin that is specific for complex N-glycans (24). The greater abundance of N-glycans on the apical surfaces of epithelial cells is clearly illustrated in Fig. 7. The fact that PHA-P is specific for complex N-glycans is confirmed by the decrease in PHA-P binding upon treatment of the cells with swainsonine, a drug that inhibits Golgi α-mannosidase II and thereby prevents the proper maturation of N-linked sugars.

DISCUSSION

Since intestinal cells are likely to be the primary targets of aerolysin upon infection of the intestinal tract by *A. hydrophila*, we investigated the effect of aerolysin on polarized epithelial cells. We found that the three cell lines we used, Caco-2,

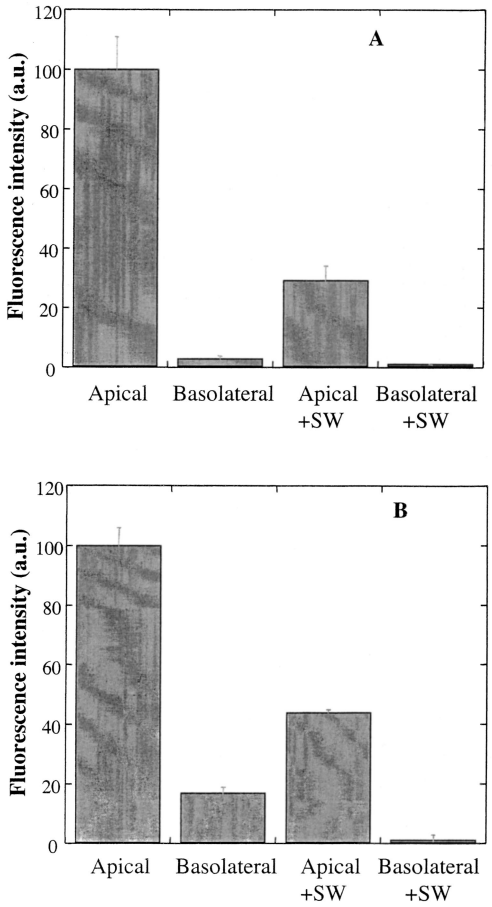

FIG. 7. N-glycans are more abundant on the apical than on the basolateral side of polarized cells. The abundance of complex N-glycans was probed by measuring binding of the N-glycan-specific fluorescently labeled lectin PHA-P on MDCK (A) and FRT (B) cells that had been treated with the Golgi mannosidase II inhibitor swainosine (+SW) or left untreated. The error bars represent standard deviations ($n = 3$). Fluorescence intensities are expressed in arbitrary units (a.u.).

MDCK, and FRT, were highly sensitive to the toxin, as expected from the ubiquitous nature of its GPI-anchored receptors. However, in contrast to what would have been predicted from the known distribution of GPI-anchored proteins, Caco-2 and MDCK cells were sensitive to aerolysin not only from the apical side but also from the basolateral side. Similarly, FRT cells were sensitive to aerolysin not only from the basolateral side but also from the apical side. Since this result was unexpected, we checked whether the effect was GPI mediated on both plasma membrane domains. PI-PLC treatment inhibited the aerolysin-induced potassium efflux by >80% in all cases. Also, on both the apical and basolateral membranes, aerolysin was associated with detergent-resistant raft-like domains, suggesting a similar mechanism for channel formation.

Although unexpected, the basolateral sensitivity of MDCK and Caco-2 cells is not inexplicable. Indeed, Lisanti et al. (32) have observed that ~90% of each detected GPI-anchored protein was found to be apically localized, suggesting that some 10% would be basolateral, which would be sufficient to render this membrane sensitive, albeit to a lesser extent than the apical membrane. The most surprising of the present observations is that the apical membranes of FRT cells are not only sensitive to aerolysin but more sensitive than the basolateral membranes, although as extensively documented (40, 41), GPI-anchored proteins are mainly basolateral (Fig. 6B, inset). This could be partly explained by the fact that aerolysin is more abundant in raft domains on the apical side of FRT cells than on the basolateral side (Fig. 5). Indeed, it has been shown that raft association promotes oligomerization and thus channel formation (4). However, the fact that four times more toxin binds to the apical surfaces even of FRT cells indicates that the event leading to increased apical sensitivity lies upstream from the oligomerization process. The greater binding could well be due to the higher abundance of N-linked sugars or other oligosaccharides on the apical surface. As recently shown by Hong et al. (24), high-affinity binding of aerolysin to its receptor requires not only the glycan core of the GPI-anchored protein but also the interaction of the lectin-like domain 1 of aerolysin (36) with an N-linked sugar on the receptor. Interestingly, it appears that this N-linked sugar must be in close proximity to the GPI anchor, possibly forming a combined binding site, since domain 1 will bind with high affinity only to N-glycans on GPI-anchored proteins. This of course does not exclude low-affinity binding of domain 1 to N-glycans on other proteins.

We propose that aerolysin initially binds to surface oligosaccharides, and in particular N-linked sugars, with low affinity before being progressively shuttled to the vicinity of the membrane, where it would bind with high affinity to the glycan core of the GPI anchor. This mode of binding would explain why even FRT cells are more sensitive to apical than basolateral exposure to aerolysin. To test this hypothesis, we analyzed the relative sensitivities of the apical and basolateral membranes of FRT cells to the alpha-toxin from *C. septicum*. This toxin shows 72% sequence similarity to aerolysin, has a similar mode of action (7), and also binds to GPI-anchored proteins (22). However, it lacks the lectin binding domain 1 found in aerolysin and is therefore unable to interact with N-glycans on its receptors and mainly binds to the glycan core of the GPI anchor (24). As predicted by our model, FRT cells bound more and were more sensitive to alpha-toxin when added from the basolateral membrane, in agreement with the distribution of GPI-anchored proteins. These observations provide the first indication of how aerolysin crosses the thick glycocalyx layer that is found on the apical side of gut epithelial cells in order to reach its high-affinity binding site, which is the glycan core of GPI-anchored proteins.

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