Biochimica et Biophysica Acta 1853 (2015) 2045–2054

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Ca^{2+}-dependent repair of pneumolysin pores: A new paradigm for host cellular defense against bacterial pore-forming toxins☆

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ARTICLE INFO

Article history:
Received 7 July 2014
Received in revised form 3 September 2014
Accepted 4 September 2014
Available online 16 September 2014

Keywords:
Plasma membrane
Calcium
Annexin
Non-immune defense

ABSTRACT

Pneumolysin (PLY), a key virulence factor of *Streptococcus pneumoniae*, permeabilizes eukaryotic cells by forming large trans-membrane pores. PLY imposes a puzzling multitude of diverse, often mutually excluding actions on eukaryotic cells. Whereas cytotoxicity of PLY can be directly attributed to the pore-mediated effects, mechanisms that are responsible for the PLY-induced activation of host cells are poorly understood.

We show that PLY pores can be repaired and thereby PLY-induced cell death can be prevented. Pore-induced Ca^{2+} entry from the extracellular milieu is of paramount importance for the initiation of plasmalemmal repair. Nevertheless, active Ca^{2+} sequestration that prevents excessive Ca^{2+} elevation during the execution phase of plasmalemmal repair is of no less importance.

The efficacy of plasmalemmal repair does not only define the fate of targeted cells but also intensity, duration and repetitiveness of PLY-induced Ca^{2+} signals in cells that were able to survive after PLY attack. Intracellular Ca^{2+} dynamics evoked by the combined action of pore formation and their elimination mimic the pattern of receptor-mediated Ca^{2+} signaling, which is responsible for the activation of host immune responses. Therefore, we postulate that plasmalemmal repair of PLY pores might provoke cellular responses that are similar to those currently ascribed to the receptor-mediated PLY effects.

Our data provide new insights into the understanding of the complexity of cellular non-immune defense responses to a major pneumococcal toxin that plays a critical role in the establishment and the progression of life-threatening diseases. Therapies boosting plasmalemmal repair of host cells and their metabolic fitness might prove beneficial for the treatment of pneumococcal infections. This article is part of a Special Issue entitled: 13th European Symposium on Calcium.

1. Introduction

Streptococcus pneumoniae (pneumococcus) causes life threatening diseases such as pneumonia, meningitis and septicemia [1]. Asymptomatic pneumococcal colonization is prevalent in the nasopharynx, but in the course of infection, the pathogen can invade sterile parts of the organism by disrupting epithelial and endothelial barriers [2]. Several pneumococcal virulence factors have been identified that mediate colonization and invasive dissemination within host tissue following pneumococcal infection [3].

Pneumolysin (PLY), a cytolysin produced by *S. pneumoniae*, is a key virulence factor of this pathogen. PLY is expressed by nearly all pneumococcal serotypes and released during bacterial autolysis or by live bacteria via mechanisms that are not yet fully understood [2,4]. PLY's principal tasks are the perforation of the plasmalemma of eukaryotic cells and the activation of complement [4]. PLY triggers numerous actions in eukaryotic cells ranging from cell death by immediate lysis or apoptosis, to cell activation via a multitude of intracellular signaling pathways and the transcriptional up-regulation of eukaryotic genes [4–8].

The cytotoxic effects of PLY are likely consequences of direct, pore-mediated effects occurring at high = lytic (cell lysis) and low = sub-lytic (apoptosis) toxin concentrations. However, the activation of eukaryotic cells occurring at sub-lytic PLY concentrations does not appear to be compatible with the toxin's pore-forming activity, which, unrestricted leads to cell death. The PLY-mediated cell activation might be brought about by recognition of PLY by Toll-like receptor 4

Abbreviations: PLY, pneumolysin; SLO, streptolysin O; TLR, toll-like receptor; PKC, protein kinase C α; HEK, human embryonic kidney 293; CCh, carbachol; AEC, airway epithelial cells; HBE, human bronchial epithelial 16HBE14o-; Anx, annexin

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http://dx.doi.org/10.1016/j.bbamcr.2014.09.005
0167-4889/

(TLR 4) [9] or other, not yet identified receptors. However, this hypothesis is not universally accepted [8].

The activation of numerous receptor-mediated signaling pathways, including TLR-dependent signaling, is controlled by the extent and duration of changes in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [10–13]. On the other hand, an elevation of $[Ca^{2+}]_i$ as a result of passive, pore-induced Ca^{2+} entry from the extracellular milieu is, presumably, the first consequence of plasmalemmal perforation due to the small size of Ca^{2+} ions and the massive gradient of $[Ca^{2+}]$ between the extracellular ($[Ca^{2+}]$ ~ 2.5 mM) and the intracellular ($[Ca^{2+}]$ ~ 100 nM) compartments.

Here we show that the detrimental effects of PLY pore-formation can be prevented by plasmalemmal repair analogous to mechanisms operative in the elimination of streptolysin O (SLO), another cholesterol-dependent cytolysin [14–17]. We further demonstrate that passive Ca^{2+} entry through PLY pores is of paramount importance for the initiation of plasmalemmal repair. Nevertheless, active Ca^{2+} sequestration that prevents excessive Ca^{2+} elevation during the execution phase of plasmalemmal repair is of no less importance. Thus, the efficacy of plasmalemmal repair depends critically on the controlled handling of the pore-induced intracellular Ca^{2+} elevation. On the other hand, in cells that were able to recover after PLY-attack, the efficacy of plasmalemmal repair defines intensity, duration and repetitiveness of PLY-induced $[Ca^{2+}]_i$ elevations that are not receptor-mediated.

Intracellular processes occurring as a result of pore formation and their elimination by cellular repair mechanisms mimic the whole palette of known receptor-mediated Ca^{2+} signals [13] and might therefore provoke PLY-induced cellular responses similar to those that are currently ascribed to the receptor-mediated cell activation by PLY [4]. Our data provide new insights into the complexity of cellular responses to a major pneumococcal toxin that plays a critical role in severe, life-threatening diseases.

2. Experimental procedures

2.1. Reagents

Living Colors Fluorescent protein vectors pECFP-N1, pEYFP-N1 and pmCherry-N1 were from Clontech and annexin constructs are described in [18]. Fluo4FF (AM) was from Invitrogen. Other reagents were from Sigma-Aldrich. PLY was prepared as described [8].

2.2. Cell culture and transfections

Human embryonic kidney 293 (HEK) cells and neuroblastoma SH-SY5Y cells were cultured as described [14]. Human bronchial epithelial 16HBE14o- (HBE) cells were maintained in MEM medium containing 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum. Dedifferentiated primary human airway epithelial cells (AEC) were kindly provided by Prof. Marianne Geiser Kamber. AEC were cultured in BEGM medium for 2 passages [19]. Cell cultures were grown in a humidified incubator at 37 °C in 5% CO_2.

HEK, SH-SY5Y and HBE cells were transiently transfected and seeded on coverslips as described [18]. AEC at passage 2 were plated on coverslips and transfected using the jetPRIME® reagent (Polyplus) according to the manufacturers' instructions. The cells were incubated for 48 h.

2.3. Imaging

Sodium Tyrode's buffer (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH = 7.4) was used in all experiments. The buffer contained 2.5 mM $CaCl_2$ or 100 μM EGTA. Transfected cells (~250,000 cells/coverslip) were mounted in a recording perfusion chamber. The cells were washed with 200 μl of Tyrode's buffer containing calcium or EGTA. At time point = 0 the buffer was removed and the cells were treated with 200 μl of purified PLY (concentrations as indicated) in Tyrode's buffer containing 10 mM dithiothreitol (DTT). Fluorescence was recorded for 45 min with an Axiovert 200 M microscope equipped with a laser scanning module LSM 510 META (Zeiss) using a 63× oil immersion lens [20].

3. Results

3.1. Visualization of plasmalemmal permeabilization by PLY pores: lysis vs. repair

Permanent permeabilization of the plasmalemma by active PLY pores results in an equilibration between soluble components of the extracellular milieu and the cytosol. Loss of the cytosol is illustrated by the leakage of cytoplasmic fluorescent protein mCherry (Fig. 1A, asterisk; Fig. S1A). The concomitant, pore-induced entry of Ca^{2+} from the extracellular milieu manifests itself in an initial increase in fluorescence of a cytoplasmic Ca^{2+}-sensitive dye Fluo4FF (Fig. 1A, asterisk; Fig. S1A). Subsequently, the fluorescence declines to background levels due to the leakage of the dye from the cytoplasm, in parallel with the loss of mCherry (Fig. 1A, asterisk; Fig. S1A).

The lytic degradation of permanently permeabilized cells culminates in the destruction of their nuclear envelope (Fig. 1B, C). Since the membrane of the nuclear envelope is permeable to small molecules, the loss of Fluo4FF from permanently permeabilized cells occurs simultaneously within nucleus and cytoplasm (Fig. 1B). However, the loss of much larger molecules of mCherry from the nucleus is significantly delayed, suggesting that the nuclear envelope remains initially intact (Fig. 1B). Failure of the nuclear envelope barrier leads to a spill of soluble nuclear components into the cytoplasm and finally, via PLY pores, into the extracellular milieu (Fig. 1C).

In contrast to lysed cells, a proportion of PLY-treated cells retain their cytosolic proteins (Fig. 1A, black asterisk; Fig. S1A). Thus, these cells are either able to prevent the assembly of functional transmembrane pores or to eliminate active pores from the plasmalemma. In cells that retain their cytoplasm, multiple transient events of elevation of Fluo4FF fluorescence are observed (Fig. 1A, graph marked by black asterisk). In line with the interpretation of similar results observed for SLO [15,20], it is very likely that this signifies a plasmalemmal perforation (pore-induced Ca^{2+}-entry) followed by the successful repair of PLY pores and the removal of excess of intracellular Ca^{2+} by the Ca^{2+} sequestrating cellular machinery as well as Ca^{2+} extrusion by the plasma membrane.

3.2. Fluorescently-labeled annexins as a read-out for plasmalemmal permeabilization, cell lysis and repair of PLY pores

In permeabilized cells, monitoring the alterations in $[Ca^{2+}]_i$ allows an almost instant detection of both pore-formation and pore-elimination events and therefore is a method of choice for studying plasmalemmal repair. However (and in contrast to SLO), transient elevations of Fluo4FF fluorescence shown in Fig. 1A (black asterisk) can also be interpreted as PLY-induced, receptor-mediated signaling that might occur even in the absence of plasmalemmal perforation [10–12].

To monitor changes in $[Ca^{2+}]_i$ that occurred exclusively as a result of PLY pore formation, we used fluorescently labeled annexins as a read-out for plasmalemmal perforation. Annexins are capable of interacting with negatively charged phospholipids in a Ca^{2+}-dependent manner [18,21,22]. In non-permeabilized cells annexins are distributed throughout the cytoplasm and within the nucleus; in perforated cells they translocate to the plasmalemma and later to the membrane of the nuclear envelope [20] (Fig. 2A; Fig. S1B). Due to their Ca^{2+}-dependent binding to cellular membranes annexins are retained even by permanently permeabilized cells (Fig. 2A, Anx A1) which have lost their soluble cytosolic components (Fig. 2A, CFP).

Different annexins require different $[Ca^{2+}]_i$ for their plasmalemmal translocation [15,18]. Whereas annexin A2 binds to the plasmalemma