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

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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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(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

of toxin-permeabilized cells already at low micromolar [Ca^{2+}], 10 μM Ca^{2+} is required for the translocation of annexin A6 and 20 μM Ca^{2+} for that of annexin A1 (Fig. 2B). The differential Ca^{2+} sensitivity manifests itself in the kinetic differences of the annexins' translocation in PLY-perforated cells: the translocation of annexin A2, which displays the highest Ca^{2+} sensitivity (see Fig. 2B), precedes that of annexin A6 and is followed by the translocation of the less Ca^{2+}-sensitive annexin A1 (Fig. 2C).

In perforated cells, the translocation of annexins closely follows the $[Ca^{2+}]_i$ elevation (Fig. 2D, PLY, the drop in the intensity of the annexin A2 cytoplasmic fluorescence is due to its translocation to the plasma membrane). The association of annexins with the plasma membrane is fully reversible: the chelation of extracellular Ca^{2+} leads to an immediate back-translocation into the cytoplasm (Fig. 2D, EGTA, time = 620 s–700 s). In the absence of extracellular Ca^{2+} the annexins leak out from permanently permeabilized cells (Fig. 2D, EGTA, time = 700 s–1000 s; Fig. S1C).

In contrast to the PLY-induced plasmalemmal translocation (Fig. 2D, PLY), even the most Ca^{2+}-sensitive annexin A2 does not react to the $[Ca^{2+}]_i$ elevation caused by the receptor-mediated activation of intracellular Ca^{2+} signaling (Fig. 2D, CCh) sufficient to trigger plasmalemmal translocation of protein kinase C α (PKC), a major component of the receptor-mediated Ca^{2+} signaling machinery (PKC; Fig. 2E). Therefore, the translocation of annexin A6, which is less Ca^{2+}-sensitive than annexin A2 (see Fig. 2B), occurs exclusively as a result of PLY-induced plasmalemmal perforation (Fig. 2F). In the permanently permeabilized cell that lost its cytosol (Fig. 2F, asterisk), annexin A6 translocated and permanently associated with the plasmalemma. In contrast, the cell that was able to repair PLY pores (Fig. 2F, black asterisk) and therefore retained its cytosol was able to restore its Ca^{2+} homeostasis, leading to annexin A6 back-translocation.

3.3. PLY pore dynamics in individual perforated cells

The annexin-based read-out for PLY-induced plasmalemmal perforation allows the investigation of plasmalemmal repair with unprecedented spatial resolution. Fig. 3A depicts a cell that successfully repaired its plasmalemmal perforations which occurred at 3 distinct plasmalemmal regions within ~25 min. An individual perforation manifests itself by local $[Ca^{2+}]_i$ elevations leading to localized translocations of cytoplasmic annexin to the damaged regions of the plasmalemma (Fig. 3A, arrows). Each of the perforations is efficiently eliminated resulting in a transient and highly localized pore-induced Ca^{2+} elevation, followed by the restoration of intracellular Ca^{2+} homeostasis and dissociation of the annexin from the plasmalemma (Fig. 3A, t = 786 s, 1167 s, 2139 s; Fig. 3B, t = 1246 s). Fig. 3B shows a cell that was able to repair lesions occurring simultaneously in several places. Fig. 3C shows two cells that completed plasmalemmal repair less efficiently, so that the pore-induced Ca^{2+} elevation spread over extended regions of cellular protrusions, whereas a cell shown in Fig. 2F (black asterisk) was able to repair the PLY-damaged plasmalemma only after the pore-induced Ca^{2+} elevation spread over the whole cell body.

3.4. Quantification of pore-induced $[Ca^{2+}]_i$ elevation

The annexin-based read-out also allows a semi-quantitative analysis of Ca^{2+} dynamics in individual cells (see Fig. 2B). Within the cell that is shown in Fig. 3A, the local, pore-induced $[Ca^{2+}]_i$ elevations reach low micromolar range (translocation of annexin A2), but remain below 20 μM (no translocation of annexin A1). Fig. 4A depicts a PLY-perforated cell, in which global translocation of annexin A2 to the

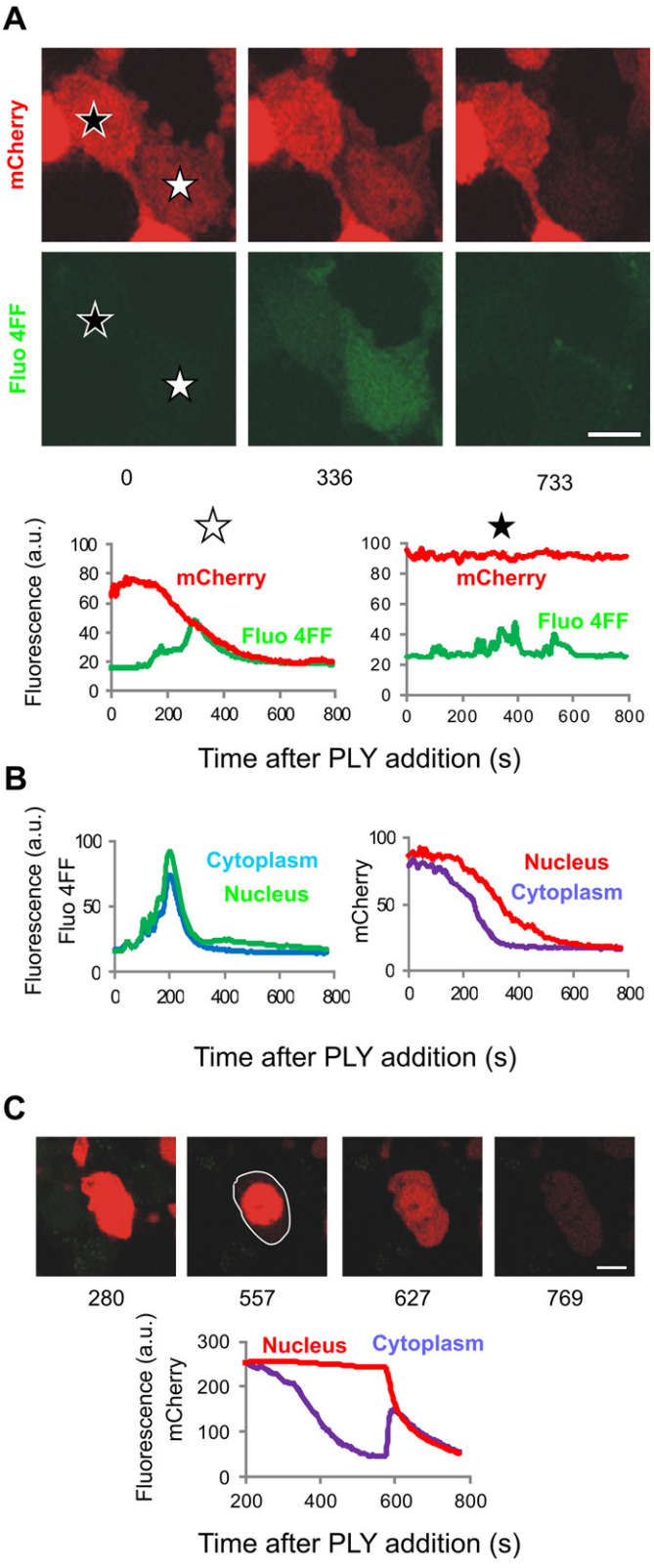

Fig. 1. Plasmalemmal permeabilization by PLY. (A, B): HEK cells transfected with cytoplasmic mCherry and loaded with Fluo4FF were challenged with PLY (4 μg/ml). (A) An increase in Fluo4FF fluorescence (green) occurs due to plasmalemmal permeabilization whereas loss of mCherry fluorescence (red) is indicative of cell lysis (top panel). Graphs (bottom panel) depict the kinetics of Fluo4FF and mCherry fluorescence within the cytoplasm of two individual cells that are marked by asterisks (black or white) in the top panel. (B) Kinetics of Fluo4FF and mCherry fluorescence were simultaneously recorded within the cytoplasm and within the nucleus of a permanently permeabilized cell. A representative recording is shown. (C) HEK cells transfected with mCherry were challenged with PLY (8 μg/ml). Kinetics of mCherry fluorescence recorded within the cytoplasm and within the nucleus of a permanently permeabilized cell. A representative recording is shown. Scale bars = 10 μm.

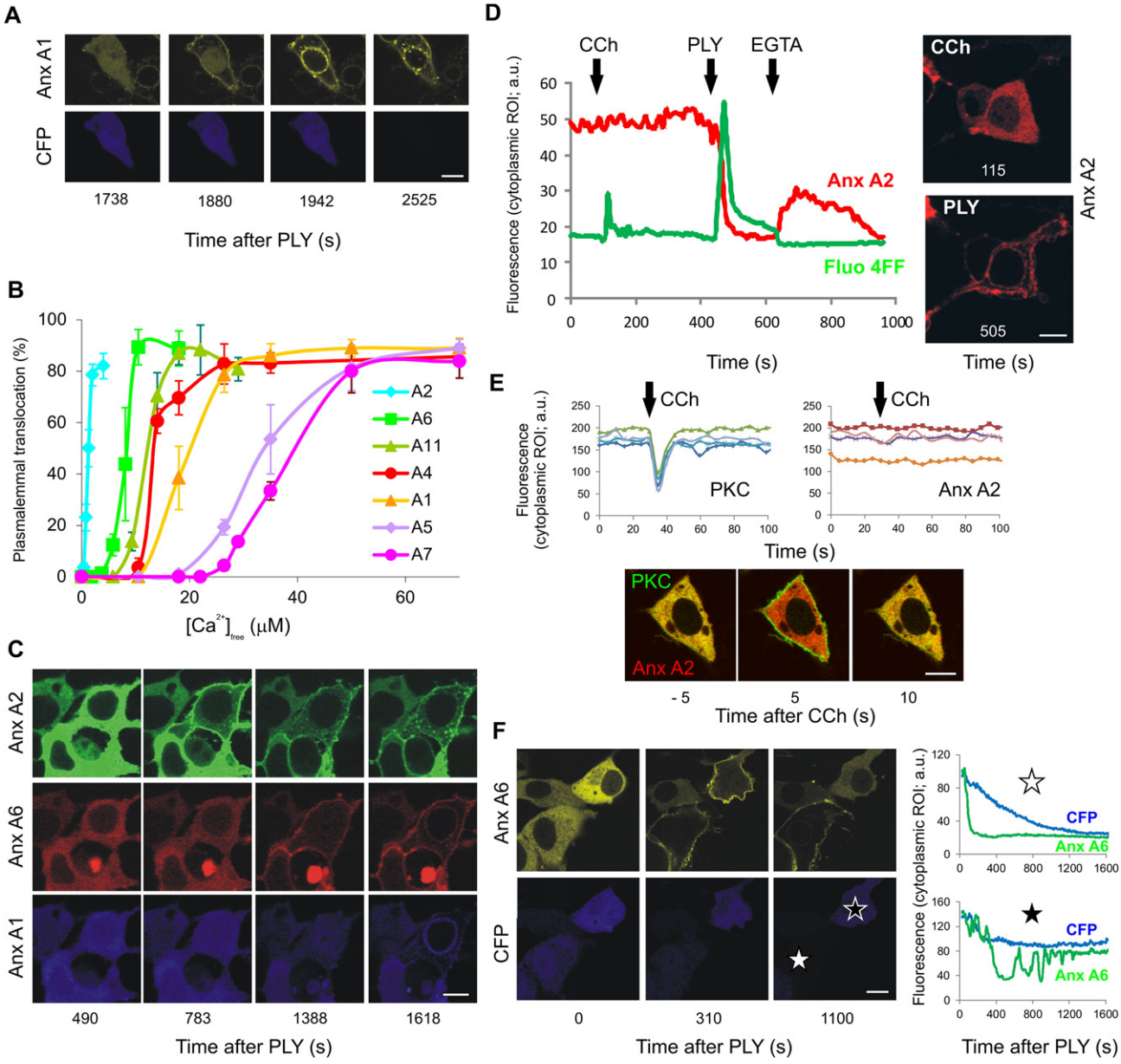

Fig. 2. Fluorescently-labeled annexins serve as a read-out for plasmalemmal permeabilization and repair of PLY pores. (A) HEK cells expressing Anx A1-YFP and cytoplasmic CFP were treated with PLY (8 μg/ml). A representative recording is shown. Anx A1-YFP remains membrane-bound even after a complete loss of cytoplasmic CFP. (B) Plasmalemmal translocations of annexins in HEK cells that were irreversibly permeabilized by lytic concentrations of SLO (14 μg/ml) in Ca^{2+}/EGTA buffers containing the indicated concentrations of free Ca^{2+} were monitored and the specific Ca^{2+}-sensitivities of plasmalemmal translocation were established for each annexin as described earlier [15]. For any experimental conditions $n \geq 7$; mean ± SEM. (C) Successive plasmalemmal translocations of Anx A2-GFP, Anx A6-mCherry and Anx A1-CFP were simultaneously visualized in HEK cells challenged with PLY (8 μg/ml). A representative recording is shown. (D) HEK cells transfected with Anx A2-mCherry were loaded with Fluo4FF and sequentially challenged with carbachol (CCh, 10 μM), PLY (8 μg/ml) and EGTA (5 mM). Kinetics of Fluo4FF and mCherry fluorescence within the cytoplasm of individual cells were recorded. A representative recording is shown. Individual images after treatment with CCh and PLY are shown for Anx A2-mCherry. (E) HEK cells expressing PKC-YFP and Anx A2-mCherry were challenged with CCh (10 μM). PKC, but not Anx A2 reacts to the CCh stimulation by plasmalemmal translocation. Representative recordings are shown. (F) HEK cells expressing Anx A6-YFP and cytoplasmic CFP were challenged with PLY (4 μg/ml). The graphs depict kinetics of Anx A6-YFP and CFP fluorescence within the cytoplasm of two individual cells that are marked by asterisks (black or white). Scale bars = 10 μm.

plasmalemma (asterisk, $t = 2244$ s) is followed by its back-translocation to the cytoplasm ($t = 3121$ s), whereas annexins A6 and A1 remain in the cytoplasm. Thus, this cell was able to repair PLY-induced damage in a manner that prevented pore-induced global elevation of $[Ca^{2+}]_i$ above 10 μM. In contrast, in the adjacent cells (Fig. 4A, bottom-right corner) the pore-induced global elevation of $[Ca^{2+}]_i$ reached values >20 μM (plasmalemmal translocation of annexin A1). Fig. 4B shows a cell, in which pore-induced global elevation of $[Ca^{2+}]_i$ reached 10 μM (plasmalemmal translocation of both annexin A2 and annexin A6, $t = 1994$ s) but was below 20 μM (cytoplasmic localization of annexin A1). Plasmalemmal repair, which was only partially successful, allowed this cell to reduce its global $[Ca^{2+}]_i$ to the low micromolar range (back-translocation of annexin A6, but not that of annexin A2, $t = 2129$ s). Fig. 4C depicts a cell experiencing PLY-induced oscillations in $[Ca^{2+}]_i$. At time points 459 s and 678 s global $[Ca^{2+}]_i$ reached low micromolar levels and at time point 1378 s it reached 10 μM. Fig. 4D shows a cell that experienced highly localized, transient elevation of $[Ca^{2+}]_i$ up to 20 μM (local plasmalemmal translocation/back-translocation of annexin A1).

3.5. Lytic PLY concentrations

The extent of $[Ca^{2+}]_i$ elevation in permeabilized cells reflects a balance between severity of plasmalemmal damage (pore-induced

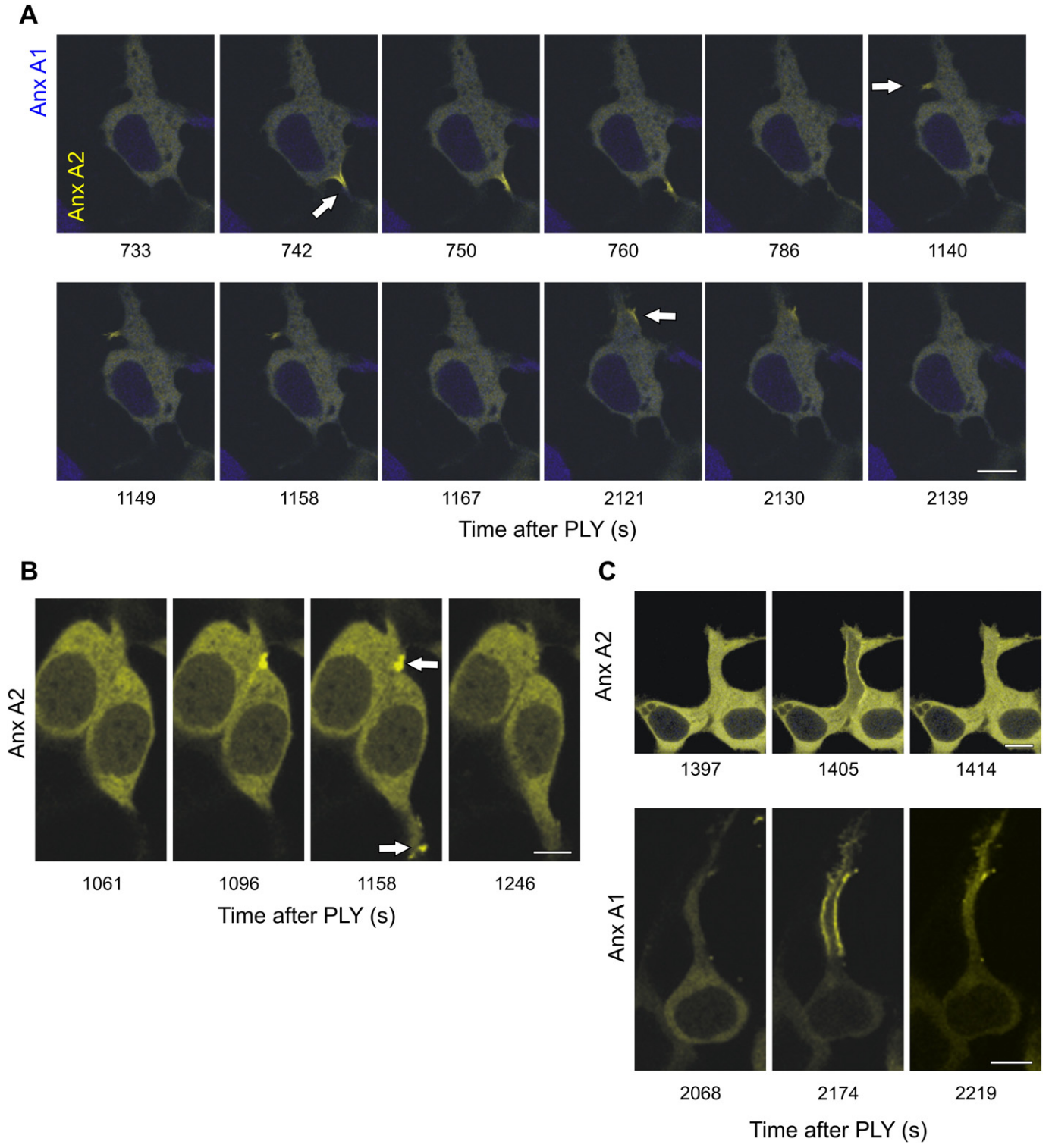

Fig. 3. PLY pore dynamics in individual perforated cells. HEK cells expressing Anx A1-CFP and Anx A2-YFP (A) or Anx A2-YFP (B, C) or Anx A1-YFP (C) were challenged with PLY (2 μg/ml). Annexin dynamics were monitored in individual cells. Annexin binding occurs at plasmalemmal sites of PLY perforation due to the influx of extracellular Ca^{2+}. Representative images are shown. Scale bars = 10 μm.

Ca^{2+}-entry) and efficiency of plasmalemmal repair (closure of transmembrane pores followed by sequestration of excessive Ca^{2+}). Therefore, to gain insight into factors that govern plasmalemmal repair, the intracellular dynamics of 3 annexins with different Ca^{2+} sensitivities of plasmalemmal translocation (annexin A2, annexin A6, annexin A1) were monitored in individual cells that were subjected to treatment with increasing PLY concentrations. Dependent on annexin dynamics, the cells were divided into 3 groups (Fig. S2): i) cells in which an annexin remained in the cytoplasm over the whole duration of the experiment were considered to be either not permeabilized or to be able to efficiently accomplish plasmalemmal repair even before the global $[Ca^{2+}]_i$ elevation reached values that supported the translocation of a

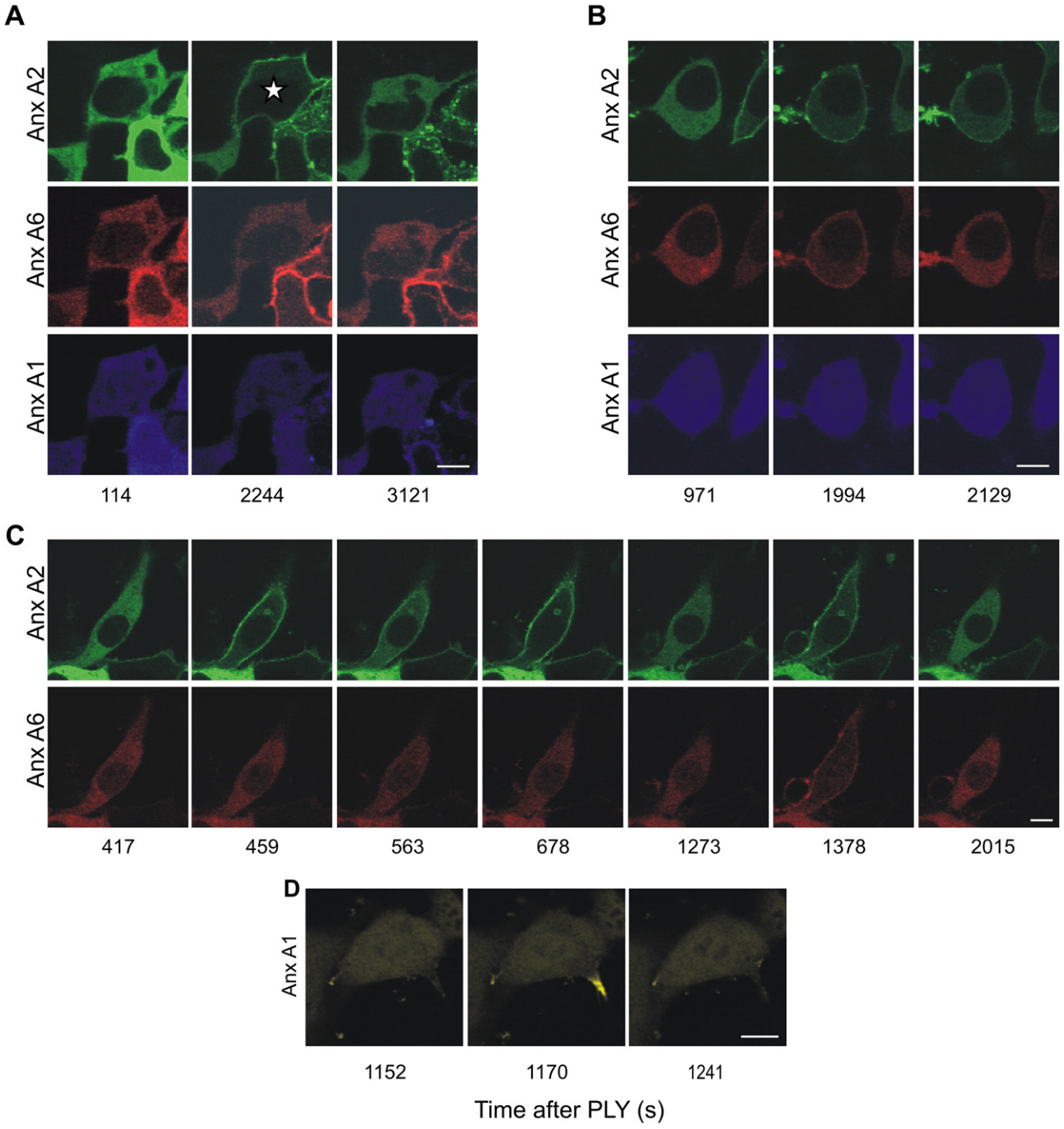

Fig. 4. Semi-quantitative analysis of pore-induced $[Ca^{2+}]_i$ elevation in individual perforated cells. HEK cells transfected with Anx A2-GFP, Anx A6-mCherry and Anx A1-CFP (A, B) or Anx A2-GFP and Anx A6-mCherry (C) or Anx A1-YFP (D) were challenged with PLY. Annexin dynamics were monitored in individual cells. The plasmalemmal binding of annexins with different Ca^{2+} sensitivities reflects $[Ca^{2+}]_i$ in PLY-perforated cells. Representative images are shown. Scale bars = 10 μm.

particular annexin; ii) cells in which at least one cycle of annexin translocation/back-translocation was observed were considered to undergo plasmalemmal repair; and iii) cells in which an annexin remained bound to the plasmalemma after initial translocation had failed to eliminate PLY pores.

Only global translocations of annexins were considered; cells displaying localized annexin-translocations such as shown in Fig. 3 were considered as efficiently repaired and were, therefore, placed in the first group. The total number of permeabilized cells irrespective of whether they were able to repair PLY pores or not was calculated by subtracting the number of non-permeabilized cells (group i) from the total number of cells in each experiment (100%).

A concentration of PLY ≥ 0.25 μg/ml was required for the permeabilization of HEK cells (Fig. 5A, squares). At 0.25–2 μg/ml [PLY] all permeabilized cells (~60% of total cells) were able to repair their plasmalemmal lesions (Fig. 5A, diamonds). A significant amount of cells that failed to repair emerged only at [PLY] above 2 μg/ml (Fig. 5A, triangles). At the highest [PLY] tested (8 μg/ml), all cells were permeabilized; ~30% of them were able to repair. A similar proportion of cells that failed to repair was observed irrespectively of whether

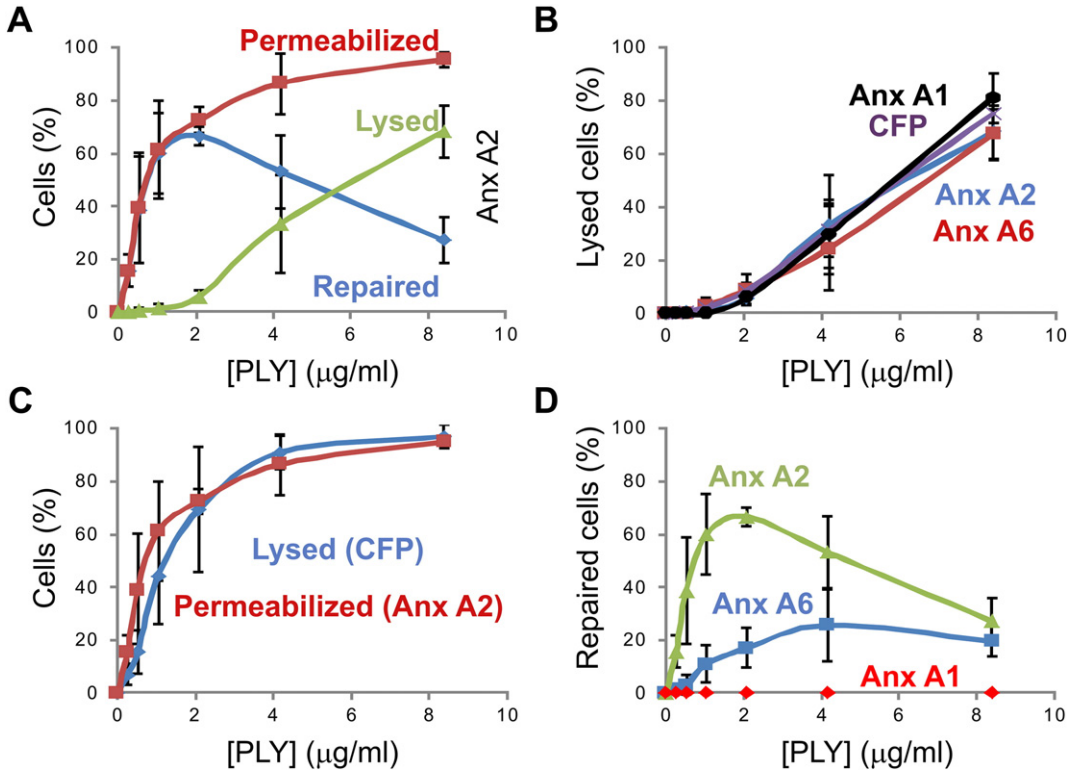

Fig. 5. Lytic [PLY] and the extent of $[Ca^{2+}]_i$ elevation in PLY perforated cells. HEK cells expressing fluorescently tagged Anx A1, Anx A2 or Anx A6 were challenged with 0.25–8 μg/ml PLY. (A) The percentage of permeabilized, lysed and repaired cells was determined as described in the text. Data for Anx A2 are shown. (B) A similar proportion of lysed cells was observed using either Anx A2, or Anx A6 or Anx A1 or CFP as a read-out. (C) In the absence of extracellular Ca^{2+} permeabilized cells are inevitably lysed. (D) Estimation of the extent of $[Ca^{2+}]_i$ elevation in repaired cells. For any experimental condition $n \geq 6$; mean ± SEM.

cell lysis was monitored using a highly Ca^{2+}-sensitive annexin A2, annexins with lower Ca^{2+} sensitivities (annexin A6, annexin A1) or a Ca^{2+}-insensitive probe (CFP) (Fig. 5B).

In the absence of extracellular Ca^{2+}, much lower [PLY] were required to lyse HEK cells (compare Fig. 5A, triangles and Fig. 5C, diamonds). At these conditions, a well characterized increase in the potency of PLY (and other cholesterol-dependent cytolysins) to lyse nucleated cells might result either from facilitated binding/assembly of PLY monomers to the plasma membrane of host cells [23] or from an inhibition of plasmalemmal repair mechanisms, which are critically dependent on the presence of extracellular Ca^{2+} [15,20]. Fig. 5C shows that the lytic curve obtained in the absence of extracellular Ca^{2+} is identical to the permeabilization curve obtained in the presence of extracellular Ca^{2+}. Thus, plasmalemmal PLY-binding and/or the assembly of active PLY pores was identical at both experimental conditions. The difference in "lytic" PLY concentrations observed in the presence or in the absence of extracellular Ca^{2+} is, therefore, exclusively defined by the activity of plasmalemmal repair mechanisms that are triggered by the entry of extracellular Ca^{2+} via PLY pores.

3.6. Intracellular Ca^{2+} elevation: point of no return

Next we analyzed the extent of $[Ca^{2+}]_i$ elevations in individual perforated cells at sub-lytic and at lytic PLY concentrations in the presence of physiological concentrations of extracellular Ca^{2+}. At sub-lytic 1 μg/ml of PLY, the majority of permeabilized cells repair plasmalemmal lesions at global $[Ca^{2+}]_i$ level of 2–10 μM (reversible translocation of annexin A2; no translocation of annexin A6), whereas the remaining permeabilized cells repair at higher, 10–20 μM Ca^{2+} levels (reversible translocation of annexin A6; no translocation of annexin A1) (Fig. 5D). At sub-lytic PLY concentrations, $[Ca^{2+}]_i$ levels never exceed 20 μM (global translocation of annexin A1) (Fig. 5D).

At lytic [PLY] the majority of repaired cells require high 10–20 μM levels $[Ca^{2+}]_i$ (reversible translocation of annexin A6) for the elimination of plasmalemmal lesions (Fig. 5D). No plasmalemmal repair was observed in cells in which $[Ca^{2+}]_i$ reached 20 μM levels (translocation of annexin A1) (Fig. 5D; Fig. S2). Thus, the global translocation of annexin A1 marks a critical phase in the fate of a permeabilized cell after which its lysis becomes imminent (point of no return). It should be noted however that localized $[Ca^{2+}]_i$ elevations of this magnitude were still tolerated by perforated cells (see Fig. 3).

The extent of $[Ca^{2+}]_i$ elevation in perforated cells is defined by the balance between pore-induced Ca^{2+} entry and the activity of intracellular Ca^{2+} sequestrating mechanisms. To establish whether $[Ca^{2+}]_i$ elevation above 20 μM merely reveals the extent of plasmalemmal damage that can no longer be repaired or if it itself plays an active role in setting the point-of-no-return in permeabilized cells, we examined PLY-induced lysis in cells that were treated with thapsigargin, an agent that inhibits the Ca^{2+} sequestrating machinery. Fig. 6A shows that cells that were treated with thapsigargin were more prone to PLY-induced lysis than untreated cells. Thus, the elevation in $[Ca^{2+}]_i$ plays a dual role in plasmalemmal repair: an increase in $[Ca^{2+}]_i$ due to Ca^{2+} entry from the extracellular milieu is of paramount importance for the activation of plasmalemmal repair mechanisms; on the other hand, the active Ca^{2+} sequestration, which prevents an excessive increase in Ca^{2+} is essential for their effective functioning. Both processes seem to be of similar importance in defining the fate of a perforated cell (Fig. 6A).

3.7. Intracellular Ca^{2+} elevation: the window of opportunity for plasmalemmal repair

Ca^{2+} homeostasis is one of the most tightly controlled intracellular processes, which is kept in balance by a coordinated activity of Ca^{2+} entry- and Ca^{2+} sequestration mechanisms; the latter being a major energy consumer. Efficient Ca^{2+} sequestration and ongoing plasmalemmal

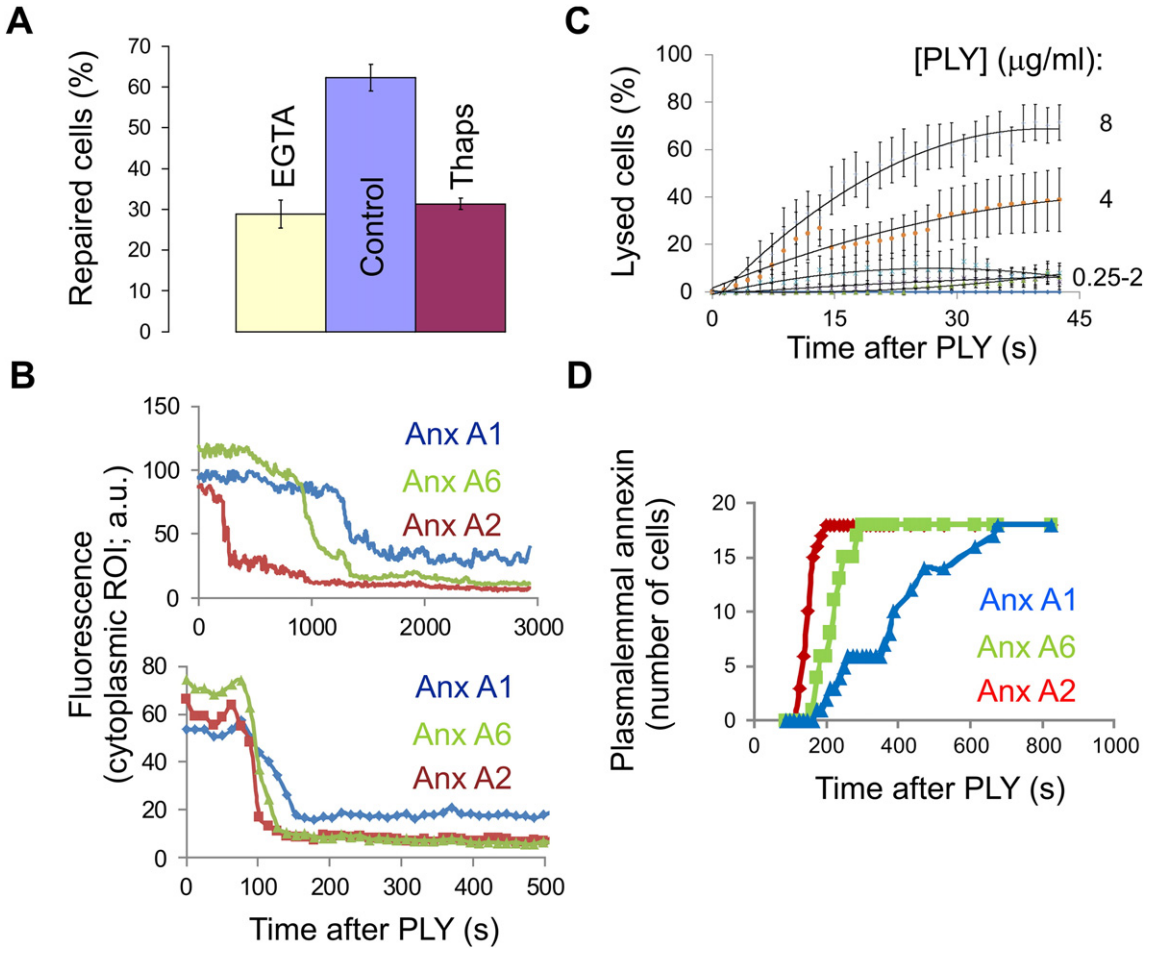

Fig. 6. Window of opportunity for plasmalemmal repair. (A) HEK cells transfected with fluorescently-labeled Anx A1 were treated with 2 µg/ml PLY (Control). Pre-treatment with 100 µM EGTA (in Ca^{2+} free Tyrode) or with 1 µM thapsigargin (Thaps) reduced the repair capacity of PLY-permeabilized cells. For any experimental condition $n \geq 10$; mean ± SEM. (B) HEK cells co-transfected with fluorescently labeled Anx A1, Anx A2 and Anx A6 were treated with PLY. Individual perforated cells significantly differ in their capability to restrict $[Ca^{2+}]_i$ elevation. (C) HEK cells transfected with fluorescently-labeled Anx A1 were treated with 0.25–8 µg/ml PLY. The percentage of lysed cells is displayed as a function of time after PLY challenge. For any experimental condition $n \geq 6$; mean ± SEM. (D) Plasmalemmal translocation of Anx A1, Anx A2 and Anx A6 was simultaneously recorded in lysed HEK cells. Whereas permeabilization occurred almost synchronously in all cells, the time which was required for the cells to succumb to the PLY injury differed significantly between individual cells.

repair are almost certainly responsible for the significant delay between cellular permeabilization (plasmalemmal translocation of annexin A2, $[Ca^{2+}]_i$ ~ 1–2 µM) and cell lysis (plasmalemmal translocation of annexin A1, $[Ca^{2+}]_i \geq 20$ µM) (Fig. 6B, upper panel). In contrast, a near simultaneous permeabilization/lysis points to an overwhelming toxin attack (Fig. 6B, bottom panel). Correspondingly, the pooled kinetics show that while lysis manifested itself within a few minutes of PLY treatment in a number of cells, the majority were still able to tolerate $[Ca^{2+}]_i < 20$ µM for up to 30 minutes (Fig. 6C). This tendency is further emphasized in the experiments shown in Fig. 6D. Here, the kinetics of the PLY-induced annexin translocations were simultaneously recorded in individual HEK cells ([PLY] = 8 µg/ml; 18 triple-transfected cells on the same coverslip). All recorded cells were synchronously permeabilized within 120 s–180 s (plasmalemmal translocation of annexin A2) of PLY treatment. An initial pore-induced elevation in $[Ca^{2+}]_i$ (~1–2 µM) was quickly and uniformly followed by a further rise in $[Ca^{2+}]_i$ up to ~10 µM (plasmalemmal translocation of annexin A6). After that, the capability to retain $[Ca^{2+}]_i$ within a range of 10–20 µM significantly varied among individual cells: whereas the 5 most vulnerable cells were lysed (plasmalemmal translocation of annexin A1) within 200 s, the 5 most resistant ones succumbed to the PLY attack only after 600–800 s. During this time the cells were able to maintain their $[Ca^{2+}]_i$ within 10–20 µM, which is in the working $[Ca^{2+}]$ range required for efficient annexin-dependent plasmalemmal repair [14,15].

Plasmalemmal repair and ensuing transient elevations in $[Ca^{2+}]_i$, similar to those described above for HEK cells, were also observed in primary human airway epithelial cells (AEC), the human neuroblastoma cell line SH-SY5Y and the human bronchial epithelial cell line 16HBE14o- (HBE), which suggest that the repair of PLY pores is a general intracellular defense mechanism that does not seem to be cell type specific (Fig. 7).

4. Discussion

PLY is a potent cytolysin of *S. pneumoniae*. In addition to the cytotoxicity, which results from direct, pore-mediated effects, PLY is able to activate host cells via a multitude of intracellular signaling pathways and transcriptional up-regulation of a large number of eukaryotic genes [7,8,24,25]. The variability of PLY effects on the host cells reflects its ambiguous role both as a virulence factor that contributes to the pathogenesis of disease as well as activator of an immune response to pneumococci [1,4,26,27]. The advantage of expressing cytolytic PLY as a virulence factor is obvious: the lysis of cholesterol-rich eukaryotic cells allows the bacteria to breach epithelial and endothelial barriers of the host and to disable its immune defenses. However, the activation of immune responses to pneumococci is not intuitively compatible with the PLY pore-forming activity, which, unrestricted, leads to the death of eukaryotic cells [27]. Therefore, the recognition of PLY at its sub-lytic

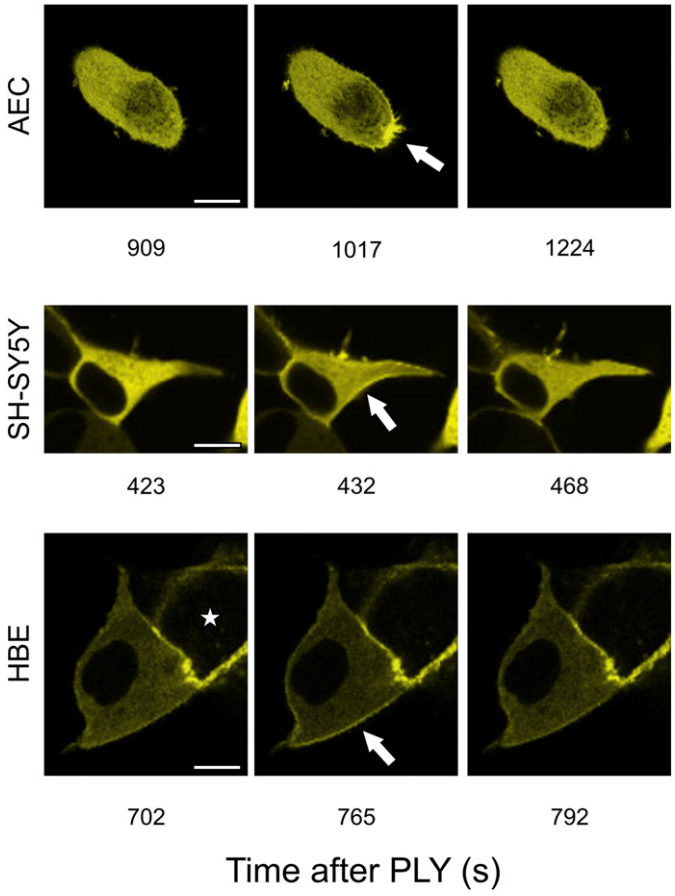

Fig. 7. PLY-induced, transient $[Ca^{2+}]_i$ elevations (indicated by Anx A2 translocation to the plasma membrane) in AEC (PLY = 16 μg/ml), SH-SY5Y (PLY = 4 μg/ml) and HBE (PLY = 2 μg/ml) cells. Scale bars = 10 μm.

concentrations by TLR4 [9] or other, not yet identified PLY-specific receptors has been suggested as a putative mechanism for the PLY-mediated activation of the host cells.

Here we provide evidence that an alternative mechanism involving PLY pore formation, followed by their elimination accompanied by the transient activation of intracellular Ca^{2+}-signaling cascades might be responsible for the activation of host cells. Plasmalemmal repair mechanisms have been extensively characterized for SLO [14–16,20]. Annexins play a critical role in plasma membrane repair by plugging and thus disabling active toxin pores [17,20]. They might also actively contribute to the subsequent shedding of the pore-containing damaged membranes [17]. An alternative repair mechanism involves lysosome-mediated patching of the pores, followed by endocytosis of the damaged membranes [28]. Our recent work suggests that shedding is responsible for the initial elimination of the toxin pores, whereas lysosome-mediated repair is indispensable for the repair of the secondary, self-inflicted mechanical damage in heavily perforated, contracting cells [14].

We show that, in analogy to the repair of SLO pores, PLY pores can be eliminated by eukaryotic cells of various origins. If a cell is able to repair its plasma membrane after PLY attack, a massive, uncontrolled increase of $[Ca^{2+}]_i$ is prevented and cellular homeostasis restored. The major beneficial outcome of plasmalemmal repair is the survival of the damaged cell; nonetheless, Ca^{2+}-dependent signaling followed by activation of PLY-targeted cells might be as important for the host.

Intracellular Ca^{2+} controls divergent cellular functions [13]. The receptor-mediated, differential activation of various signaling pathways depends on the extent and duration of calcium signaling patterns that can occur as single transients, repetitive oscillations or sustained plateaus [13,29]. Similar signaling patterns, albeit of much higher amplitude, were observed in repairing epithelial HEK cells that lack the putative PLY receptors TLR2 and TLR4 [30] and also in cultured cells of other origins. At sub-lytic PLY concentrations the amplitude of $[Ca^{2+}]_i$ in the majority of permeabilized cells was kept between 2 and 10 μM; in a smaller population of cells $[Ca^{2+}]_i$ above 10 μM, but inevitably below 20 μM. At lytic PLY concentrations, the majority of cells that were able to withstand a PLY attack experienced $[Ca^{2+}]_i$ in the 10–20 μM range. Since the activation of cytokine production can be mediated solely through an increase in $[Ca^{2+}]_i$ [31,32], it is conceivable that transient Ca^{2+}-signals occurring in PLY-perforated cells as a result of pore elimination by plasmalemmal repair are recognized by the host as a signal for the presence of a pathogen and are responsible for the activation of host immune responses to pneumococcal invasion.

At any PLY concentration that was tested, the total population response was never uniform: there were always cells that resealed efficiently restricting their Ca^{2+} elevations to short transients and cells that were exposed to oscillations or long plateaus of high Ca^{2+} concentrations. Moreover, a cycle of plasmalemmal perforation/plasmalemmal repair often led to highly localized $[Ca^{2+}]_i$ elevations of high magnitude within individual cells. It is also plausible that due to the fast diffusion of small Ca^{2+} ions within the cytoplasm, a gradient of elevated $[Ca^{2+}]_i$ forms within a single perforated cell. Such inhomogeneity within a population of PLY exposed cells or even within single cells might lead to an uncoordinated, simultaneous activation of Ca^{2+} signaling pathways, which are characterized by different Ca^{2+} sensitivities. This stands in contrast to the Ca^{2+} signaling evoked by the stimulation of specific receptors that are typically homogeneously expressed throughout a cell population in a highly regulated manner and evenly distributed within the cellular plasma membrane. It is conceivable that at high [PLY], such an unregulated activation of targeted cells that experience a prolonged $[Ca^{2+}]_i$ elevation of pathological scale might be responsible for the development of the overactive immune response.

The effective targeting of annexins to plasmalemmal pores is triggered by the localized $[Ca^{2+}]$ elevation within the cytoplasmic region directly adjacent to the toxin-damaged membrane. However, if plasmalemmal repair cannot be completed in an efficient way, further pore-induced $[Ca^{2+}]_i$ elevation might provoke global annexin binding even to the non-damaged plasmalemma. Such non-productive binding prevents the annexins from executing their repair functions [15]. Thus, the annexin-dependent plasmalemmal repair can be executed only during a period which commences with pore-formation (localized Ca^{2+} elevation) and finishes once the global $[Ca^{2+}]_i$ level reaches a threshold at which non-productive binding of an annexin to the undamaged plasmalemma will drastically deplete its free, repair-capable cytoplasmic pool. Our data suggest that the upper limit of global $[Ca^{2+}]_i$ elevation that can be tolerated by a perforated HEK cell is ~20 μM. This corresponds to the plasmalemmal translocation of annexin A1. Such a correlation might be accidental, since annexins A5 and A7 possess an even lower Ca^{2+} sensitivity (see Fig. 2B). On the other hand, it is plausible that the point-of-no-return in HEK cells is indeed defined by the non-productive annexin A1 binding to the undamaged plasmalemma, since annexins with an even lower Ca^{2+} sensitivity initially leak out from the perforated cells to support plasmalemmal repair from the outside [33]. In HEK cells, annexin A1 might be largely responsible for the plasmalemmal repair occurring at lytic PLY concentrations, whereas annexins A2 and A6 accomplish pore-elimination at sub-lytic [PLY].

Our results indicate that the window of opportunity for plasmalemmal repair is defined by the intrinsic properties of a prevalent annexin that is expressed in a particular cell type. The role of keeping this window open falls to intracellular Ca^{2+} sequestrating mechanisms. Due to limited capacity of cellular organelles, sequestration can provide only temporary protection against cytosolic Ca^{2+} elevation induced by Ca^{2+} influx. It however could prevent the development of store operated Ca^{2+} entry (SOCE), which could aggravate the situation. Plasmalemmal and reticular Ca^{2+} pumps that are largely responsible for the removal

of the excess of Ca^{2+} ions from the cytoplasm are also major consumers of cellular ATP. It is therefore conceivable that plasmalemmal permeabilization by PLY leads to a preferential elimination of the metabolically least fit cells, thereby contributing, after resolution of the infection to an improved overall fitness of the total host cell population.

Recognition of the ability of nucleated cells to repair PLY pores adds to our understanding of bacteria–host interactions. The chances of a host cell to be terminally damaged by PLY are not only defined by the toxin's proficiency to bind and permeabilize the cellular plasma membrane but also by the capability of the host cells to eliminate PLY pores and actively retain their homeostatic balance despite a breakdown of the plasmalemmal permeability barrier. It also appears that the activation of Ca^{2+} signaling cascades due to the combined outcomes of plasmalemmal permeabilization and plasmalemmal repair can be used by the host for the recognition of bacterial invasion and for the mounting of a protective immune response. However, in heavily damaged cells that survive the PLY-attack, $[Ca^{2+}]_i$ can reach elevated non-physiological levels provoking a dysregulation of their homeostasis leading to an uncontrolled immune reaction.

Our findings suggest that host-centered, therapeutic strategies that target the plasmalemmal repair of host cells [14,34] and boost their general metabolic fitness might prove to be beneficial for the treatment of pneumococcal infections.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2014.09.005.

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