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Commentary

Plasma membrane repair and cellular damage control: The annexin survival kit

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ABSTRACT

Plasmalemmal injury is a frequent event in the life of a cell. Physical disruption of the plasma membrane is common in cells that operate under conditions of mechanical stress. The permeability barrier can also be breached by chemical means: pathogens gain access to host cells by secreting pore-forming toxins and phospholipases, and the host's own immune system employs pore-forming proteins to eliminate both pathogens and the pathogen-invaded cells. In all cases, the influx of extracellular Ca^{2+} is being sensed and interpreted as an "immediate danger" signal.

Various Ca^{2+}-dependent mechanisms are employed to enable plasma membrane repair. Extensively damaged regions of the plasma membrane can be patched with internal membranes delivered to the cell surface by exocytosis. Nucleated cells are capable of resealing their injured plasmalemma by endocytosis of the permeabilized site. Likewise, the shedding of membrane microparticles is thought to be involved in the physical elimination of pores. Membrane blebbing is a further damage-control mechanism, which is triggered after initial attempts at plasmalemmal resealing have failed.

The members of the annexin protein family are ubiquitously expressed and function as intracellular Ca^{2+} sensors. Most cells contain multiple annexins, which interact with distinct plasma membrane regions promoting membrane segregation, membrane fusion and – in combination with their individual Ca^{2+}-sensitivity – allow spatially confined, graded responses to membrane injury.

It is a truth universally acknowledged that the health and well-being of a cell critically depend on the integrity of its plasma membrane. Yet, depending on their specific task or their localization within the organism, many cells are likely to experience membrane injury on a frequent and recurring basis. Indeed, it can be considered natural for a cell to undergo membrane injury if placed in harm's way. Epithelia, especially those of the gastrointestinal tract and lung are subject to continual mechanical disruption, the endometrial lining of the uterus undergoes regular cycles of destruction and repair, vascular endothelial cells are prone to suffer damage through oxidized lipoproteins, membrane fissures are the result of mechanical strain in skeletal muscle cells and immune cells are attacked by toxins secreted by invading pathogens or by the host's own defence systems such as perforins and blood complement complexes [1–6].

The lipid bilayer of the plasma membrane constitutes a barrier, which maintains the essential differences between the cytosol and the extracellular environment. Any disruption of this bilayer leads to an uncontrolled influx of extracellular Ca^{2+}, often accompanied by an efflux of cytoplasmic constituents. An abrupt, uncontrolled rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) triggers degenerative biochemical and structural events and thereby causes cell death. It is therefore a good idea for a potentially vulnerable cell to keep a well-appointed repair toolkit ready for emergencies.

1. Membrane repair: general mechanisms

Nucleated cells survive the disruption of their plasma membrane by a process of resealing [7]. "The recovery of an injured cell is only brought about by the formation of a membrane-like film which prevents extension of the injury" states an early observer of marine invertebrate ova, which he subjected to needle injury [8]. Membrane resealing was originally thought to be a passive process, automatically mediated by the spontaneous reorganisation of phospholipids into their thermodynamically most stable state (Fig. 1A) [7]. Developed by investigating the repair process of liposomes [9], this concept turned out to be incompatible with live cells, since membrane tension and a subcortical cytoskeleton oppose spontaneous resealing. Under these conditions the repair of lesions of more than 0.2 μm in diameter cannot realistically be achieved. Thus, larger lesions require either active plasma membrane replacement, which might

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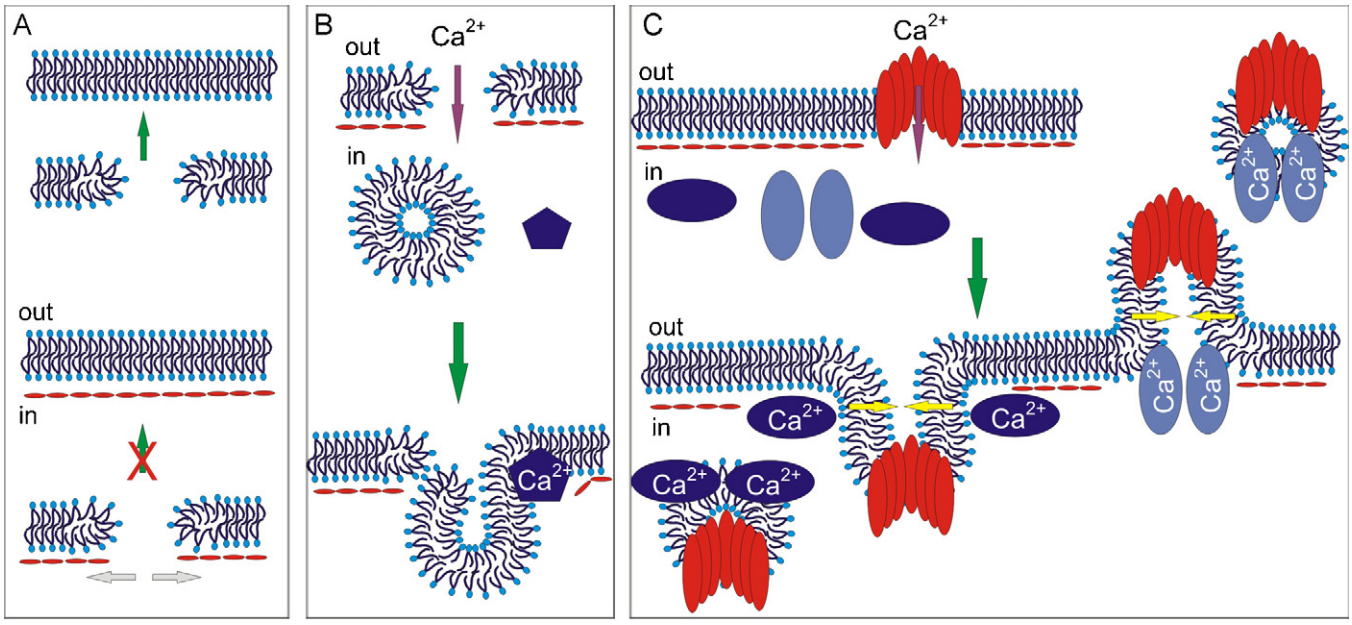

Fig. 1. Schematic representations of different membrane repair mechanisms. (A) Spontaneous membrane resealing. Upper panel: self-sealing by spontaneous lipid flow across the injured site within a liposomal bilayer. Lower panel: in live cells, spontaneous resealing is prevented by contraction of the subcortical cytoskeleton (red) and thereby exerts forces in opposing directions. (B) Plasma membrane resealing by exocytosis: a plasmalemmal lesion (upper panel) causes an influx of Ca^{2+} from the extracellular space, which triggers the fusion of a lysosomal vesicle by means of a Ca^{2+}-dependent fusogenic protein (blue pentagon) with the plasma membrane (lower panel), and forms a "patch" across the damaged site. (C) Membrane repair by endocytosis and microparticle shedding. Upper panel: A toxin-induced transmembrane pore (red) is assembled within the plasma membrane. Lower panel: the influx of Ca^{2+} activates proteins, which either induce endocytic uptake of the pore (left) or outward budding and vesiculation of the injured site (right).

be provided by the fusion of intracellular vesicles with the plasma membrane or the damaged sites must be physically removed from the plasmalemma [7].

The fusion of intracellular vesicles (Fig. 1B) was initially identified as the mechanism operative in the repair of the sea urchin egg membrane following mechanical injury [10]. Electrophysiological evidence suggested an involvement of the lysosomal pathway in plasmalemmal resealing [11] and studies of cell invasion by *Trypanosoma cruzi* confirmed lysosomal fusion with the plasma membrane [12]. The "patch" hypothesis implicates the initial fusion of multiple lysosomes into a giant, pre-formed patch vesicle before its exocytic fusion with the damaged plasmalemma [7]. Implicated in plasma membrane repair and endowed with properties for membrane fusion, but distinct from lysosomes, is the enlargosome: an exocytotic compartment, characterized by the expression of the large membrane protein AHNAK/desmoyokin [13,14]. However, the repertoire of the intracellular membranes that are involved in "patch"-resealing is probably not limited to the two above-mentioned organelles [15]. The course of membrane resealing in experimentally wounded sea-urchin eggs suggest that even secretory granulae might be recruited to patch a lesion [7]. Thus it appears that when it comes to the worst, a cell forcibly conscripts any available source of intracellular membranes without regards for its function in everyday life. "Patching" appears to be the predominant mechanism operative in mechanically induced lesions containing "free lipid edges" which are ready to be fused with the lipids of the intracellular vesicles.

On the other hand, physical removal of the damaged plasmalemmal regions seems to be the strategy of choice to mend the stable, protein-lined membrane lesions caused by pore-forming proteins [16–19]. Lesions formed by either bacterial toxins or by the proteins of the membrane attack complex (MAC) of blood complement induce an outward vesiculation of the membrane regions containing the proteinaceous pores, followed by their consequent shedding into the extracellular milieu (Fig. 1C).

The generation of a MAC is held to be a defense mechanism of the immune system. It consists of 5 complement proteins (C5–C9) assembling into a ring structure within the plasma membrane, which allows the free diffusion of molecules into and out of the cell [20]. Microparticle shedding represents the major route of MAC removal in human neutrophils which were targeted with a sub-lytic amount of homologous complement: 65% of the bound MACs were rapidly removed from the cell surface on vesicles representing just 2% of the cell surface [21]. Several other cell types have subsequently been shown to remove MACs by vesiculation/shedding, e.g. human platelets, rat glomerular cells, human synoviocytes and rat oligodendrocytes [16].

In contrast to shedding, neutrophils remove only a small proportion of cell-bound MACs by endocytosis (Fig. 1C) and subsequent intracellular degradation [21]. However, in other cell types, endocytosis appears to be the primary route of MACs elimination [16]. Likewise, endocytosis seems to be responsible for the elimination of the pores created by the bacterial toxin streptolysin O (SLO) in HeLa and NRK (normal rat kidney) cells [18]. In contrast, human embryonic kidney (HEK 293) cells [19] and human fibroblasts [22] neutralise SLO-pores by shedding.

It is conceivable that both mechanisms of physical pore removal operate simultaneously within a single cell; their relative importance, however, may differ between cell types and might also be dependent on the mode of injury. Moreover, recent experiments suggest a co-operation between patch- and endocytic events which are taking place simultaneously during membrane resealing [23].

2. Elevation of intracellular Ca^{2+} concentration and the fate of perforated cells

In stark contrast to the spontaneous membrane resealing in liposomes, a rise in $[Ca^{2+}]_i$ is critical for successful plasma

membrane repair and the recovery of nucleated cells [7,24]. At the same time an intracellular Ca^{2+}-overload is held responsible for the death of pore-bearing cells [25]. And a limited Ca^{2+}-influx followed by transcriptional activation is thought to induce a variety of biological responses associated with the sub-lytic effects of pore-forming toxins [16,17,26]. Thus, it appears that the extent of $[Ca^{2+}]_i$ elevation following pore formation determines the fate of a targeted cell. Consequently, Morgan et al. [25] suggested that in MAC-perforated cells an initial increase in $[Ca^{2+}]_i$ stimulates the recovery processes, allowing the cell to withstand a limited complement attack. The recovery can be associated with cellular activation and the production of inflammatory modulators, which in turn amplify an ongoing inflammatory response [25]. The authors further hypothesised that more severe membrane damage causes a sharp rise in intracellular $[Ca^{2+}]_i$ which overwhelms all recovery processes [25].

Similarly, the fate of SLO-perforated cells is dependent on their ability to control the extent of a pore-induced elevation $[Ca^{2+}]_i$ [19]. Plasmalemmal repair, operating in the 5–10 μM range of $[Ca^{2+}]_i$, leads to the recovery of perforated HEK 293 cells that eliminate the toxin-induced pores by shedding them in form of extracellular microparticles [19]. However, the plasma membrane repair mechanisms are unable to prevent irreversible cell damage if $[Ca^{2+}]_i$ rises above 10 μM. Under these conditions, persistent changes in the lipid composition and in the architecture of the plasmalemma, induced by the Ca^{2+}-dependent formation of ceramide platforms, drive the injured cells beyond a point at which recovery is still possible [27]. Similar to MAC-perforated cells, the fate of SLO-permeabilized cells is not limited to two mutually exclusive options: cells, which are capable of preventing an elevation of $[Ca^{2+}]_i$ above the critical concentration, yet are unable to complete plasma membrane repair, enter a prolonged phase of $[Ca^{2+}]_i$ oscillations. $[Ca^{2+}]_i$ oscillations have been shown to induce a sustained cell activation which is followed by proliferation, differentiation or release of biologically active substances [28]. In contrast to a physiological stimulation, the pore-induced cell activation is beyond the organism's control and potentially harmful. Thus non-lethal effects of pore-forming cytotoxic agents which are brought about by $[Ca^{2+}]_i$ oscillations may be as important to health and disease as their extensively documented lytic properties.

3. Repair toolkit

The requirement of elevated Ca^{2+} for successful plasmalemmal repair [7] is further emphasized by the fact that most of the proteins, which are implicated in membrane resealing are either Ca^{2+}-sensors themselves or their activity is regulated by Ca^{2+}-sensors. Molecules which prominently feature in membrane repair events belong to the families of synaptotagmins, SNAREs, ferlins, and annexins. Recent data also implicate MG53, a member of the TRIM (tripartite motif) protein family in the repair of skeletal muscle sarcolemma [29].

Mechanisms controlling membrane damage and repair are universal, hence, analogues of synaptotagmins, SNAREs and annexins have been identified in plants (see below). A ferlin analogue has not yet been detected [30]. An overview is presented in Table 1.

4. Synaptotagmins, SNAREs and ferlins

Synaptotagmin 1 was initially identified in a proteomic screen for molecules responsible for transmitter release [31] and shown to be a member of a family of structurally related proteins, which are characterised by an NH_2-terminal transmembrane domain, a linker region and two COOH-terminal C2 domains [32]. C2 domains are independent folding units, which are responsible for mediating Ca^{2+}-dependent phospholipid binding [32]. Currently most, if not all, proteins that contain C2 domains are thought to interact with cellular membranes. However, the second synaptotagmin C2 domain interacts Ca^{2+}-independently with clathrin, suggesting that some C2 domains may play a purely structural role or may have lost the ability to bind Ca^{2+} [33]. Apart from neurotransmitter release, synaptotagmins orchestrate other exocytotic events such as the secretion of insulin [34].

Synaptotagmin 1 was the first plant synaptotagmin characterised in *Arabidopsis thaliana* as a cold-responsive plasma membrane protein [35]. Schapire et al., [30] suggested that the repair mechanism mediated by synaptotagmin 1 involved the Ca^{2+}-dependent fusion of vesicles with the plasma membrane. A loss-of-function mutant in *Arabidopsis thaliana* showed diminished resistance to freezing, salt and drought stress, resulting in a general decrease in plant fitness that was enhanced under non-optimal growing conditions [30]. Thereafter, several studies confirmed its role in the maintenance of membrane integrity in plants subjected to stressful conditions such as osmotic stress or freezing [36].

The identification of synaptotagmin VII as an animal lysosomal synaptotagmin shed light on the molecular mechanism determining the role of lysosomes in membrane repair [37].

Synaptotagmins operate in concert with the membrane fusion proteins of the SNARE family [38]. SNAREs exist on vesicle

Table 1
Plasma membrane repair: proteins and mechanisms.

Proteins involved	Cell type	Injury/defect	Mechanism of membrane repair	References
Synaptotagmins	Plant cells	Cold injury	Membrane fusion	[30,35,36]
	Murine fibroblasts	Contraction	Exocytotic fusion	[37]
SNAREs	Plant cells	Drought	Unknown	[45]
	Sea urchin embryos	Laser injury	Exocytotic fusion	[41]
Synaptotagmin and SNAREs	Normal rat kidney	Mechanical injury	Exocytotic fusion	[43]
	Normal rat kidney	Pore-forming toxin; mechanical injury	Exocytotic fusion/endocytosis	[44]
Ferlins	Skeletal muscle (dysferlin)	Laser injury	Exocytotic fusion	[50]
Annexins	Plant cells (AnnAt1)	Stress related	Unknown	[92,93]
	HeLa	Mechanical, Laser injury	Exocytotic fusion	[80]
	HEK 293	Pore-forming toxin	Microparticle shedding	[19]
Annexins and Ferlins	Skeletal muscle (dysferlin, annexins A1 and A2)	Mechanical injury	Exocytosis	[46,79]
MG53 and dysferlin	Skeletal muscle	Laser injury	Exocytotic fusion	[29]

membranes (vSNAREs) and target membranes (tSNAREs), which form pairs in order to effect heterotypic membrane fusion, but in addition, SNAREs are instrumental in homotypic fusion events [39]. SNARE proteins are intimately involved in all steps of vesicle trafficking: the SNARE-mediated trafficking of the glucose transporter 4 (GLUT-4) is a well characterized example [40]. In sea urchin eggs, which were injected with botulinum toxin A known to cleave SNAREs, resealing was shown to be inhibited [41]. Yet it appears that additional molecules are needed in order to organize a functional SNARE complex, since the complex does not possess specific Ca^{2+}-binding sites [42]. Thus it is incapable of assembling its constituents and translocating to the site of injury with the speed needed for spontaneous membrane repair or rapid vesicle secretion. For this reason SNAREs have been thought to team up with the synaptotagmins, which provide the Ca^{2+}-sensitivity necessary for rapid reaction and complex assembly. Synaptotagmin–SNARE protein interactions regulate the lysosomal, Ca^{2+}-triggered exocytosis during membrane repair in normal rat kidney cells [43]. The following sequence of events occurs during this process: an injury-induced Ca^{2+}-influx is sensed by lysosome-associated synaptotagmin VII (Syt VII); Ca^{2+}-binding triggers Syt VII association with membrane phospholipids and the tSNAREs allowing the formation of a complex between tSNAREs and vSNAREs, which mediates the lysosomal fusion with the injured region of the plasma membrane [43] [44].

Plant analogues of SNAREs have been identified in tobacco and *Arabidopsis thaliana* in a screen for signalling elements associated with drought [45].

Displaying a similar protein structure as the synaptotagmins and comparable functional properties, the ferlin protein family occupy a prominent place in Ca^{2+}-sensing and plasma membrane repair [46,47]. To date, six members of the ferlin gene family have been identified in most vertebrates (FER1L1-6) [47]. They are evolutionarily ancient proteins, which contain highly conserved six tandem C2 domains and a COOH-terminal transmembrane domain [47]. This unusually high number of C2 domains suggests that not all of them are responsible for Ca^{2+}-dependent phospholipid-binding. It is likely that – in analogy to the synaptotagmins – some C2 domains have undergone a shift in their functional properties or acquired additional functions in protein–protein interactions, small molecule binding and signalling [33] [47].

Mutations in ferlins (otoferlin and dysferlin) have been linked to inherited diseases [47]. Otoferlin is a regulator of SNARE-mediated membrane fusion [48] and a Ca^{2+}-sensor in cochlear hair cells. Its mutations are associated with deafness [49]. Dysferlin, which is prominently expressed in skeletal and cardiac muscle sarcolemma, has been credited with a membrane "maintenance" function [46].

Since contraction–relaxation cycles put a high amount of strain on skeletal muscle fibres, a rapid task-force for miniature fissures is indispensable. The sarcolemma of structurally injured muscle fibres is enriched in dysferlin, which suggests that diminished membrane repair mechanisms are responsible for the gradual, exercise-linked destruction of skeletal muscle cells observed in patients affected by dysferlin-associated myopathies. The interaction of dysferlin with phospholipids and the presence of multiple Ca^{2+} binding sites, suggest its involvement in vesicle recruitment and heterotypic membrane fusion [46,50,51]. Dysferlin mutations lead to a reduction in dysferlin protein and its irregular distribution within the sarcolemma in patients with limb-girdle muscular dystrophy 2B (LGMD2B), or Miyoshi muscular dystrophy [52]. A recent report has linked dysferlin to Ca^{2+}-triggered, paracrine intercellular signalling from wounded to intact sea urchin embryos cells via the release of ATP [53]. These signals enable the neighbouring cells to prepare for the potential loss of an adjoining cell.

5. Annexins

The annexins are a multigene family of Ca^{2+}- and phospholipid-binding proteins [54]. They are structurally related and expressed in most phyla and species. Twelve annexins are present in vertebrates (A1-A11 and A13) which display different splice variants [55]. Their COOH-terminal core is evolutionarily conserved and contains phospholipid- and Ca^{2+}-binding sites; their NH_2-terminal tail is unique and enables the protein to interact with distinct cytoplasmic partners. The annexin domain responsible for Ca^{2+}- and phospholipid binding is not identical with the C2 domain. Instead, the annexin–membrane interaction occurs via the formation of a ternary complex between annexin, Ca^{2+} and the negatively charged phospholipids of the membrane [56].

In addition to the synaptotagmins and the ferlins, the annexin protein family constitutes yet another internal Ca^{2+}-sensor: an elevation of $[Ca^{2+}]_i$ acts as a trigger for the translocation of annexins from the cytosol to the plasma membrane. Each annexin requires a different and individual level of free calcium for its translocation to cellular membranes. Thus, several annexins present within any one cell – each with its distinct Ca^{2+}-threshold for membrane translocation – form a broad-range Ca^{2+}-sensing system [57–59]. The Ca^{2+} sensitivity of annexin–membrane binding is modulated by their interaction with other proteins [54,59] and proteolytic cleavage [60].

Annexins have been credited with a bewildering array of functions [54]. However, the very first paper ever to appear on annexins reported vesicle aggregation as a characteristic feature of "synexin", which is now called annexin A7 [61]. Vesicle association and involvement in vesicular transport have been shown for several annexins: annexin A2 is instrumental in intracellular vesicle movement [62] and is associated with endosomal functions [63]. An endosomal localization has also been reported for annexin A1 [64,65]. Annexin A5 has previously been associated with late endosomes [66]. Annexin A6 has been associated with EGF receptor signalling and endocytic transport [67]. Annexins A1, A2 and A6 play a role in the regulation of endocytic membrane traffic and in the biogenesis of multivesicular bodies [68].

The initiation of developmental activities in the ova of many species is triggered by transient increases in Ca^{2+} during fertilization [51]. Starting at the site of sperm entry, a Ca^{2+} wave with a peak of up to 30 μM traverses the cytoplasm and triggers the cortical reaction [29,69]. Osterloh et al. have cloned four annexins from the killifish *Oryzias latipes*, which were already present in unfertilized eggs and which were expressed in a developmentally regulated manner after fertilization [70]. One of those annexins was shown to induce the aggregation of membrane vesicles at micromolar levels of Ca^{2+}, which renders it a suitable candidate for participation in the cortical reaction [71].

The fusogenic properties of the annexin protein family have been held responsible for the interaction of annexin A2 with members of the SNARE family in the exocytosis of chromaffine granules and in the secretion of pulmonary surfactant [72–74]. Purified or endogenous annexin A2 derived from type II alveolar epithelial cells co-immunoprecipitated and specifically bound with SNAP-23, a member of the SNARE-family, in a Ca^{2+}-dependent manner [73].

6. Annexins as an experimental tool to study plasmalemmal injury

An obvious sign for plasmalemmal damage is the externalization of phosphatidylserine to the outer leaflet of the plasmalemma.

The permanent loss of lipid asymmetry during apoptosis or after plasmalemmal injury can be directly monitored by binding of tagged, extracellularly added annexin A5 to the surface of the affected cells [75,76]. Recent reports demonstrate that the externalization of phosphatidylserine can be a transient process, which occurs as a consequence of cell stress or ischemia of brief duration after which the surface-bound, labelled annexin A5 is internalized by endocytosis [77]. After a single period of ischemia, injured and successfully repaired cardiomyocytes can so be identified with high sensitivity [77]. Thus, the presence of tagged-annexin A5 on the surface of the plasma membrane or within endosomes indicates that a permanent or temporal interruption in the continuity and/or the asymmetry of the lipid bilayer has taken place.

Intracellular $[Ca^{2+}]$ elevation plays a crucial role in determining the outcome of plasmalemmal injury. However, in permeabilized cells, the quantitative evaluation of $[Ca^{2+}]_i$ with standard approaches including the use of Ca^{2+}-sensitive fluorescent dyes is problematic due to the leakage of the cytoplasmic dye. In contrast, the Ca^{2+}-sensitive annexins are retained by a permeabilised cell due to their binding to the plasmalemma. Recently, we have shown that following $[Ca^{2+}]_i$ elevation four different annexins translocate to the plasmalemma, each at a different $[Ca^{2+}]_i$ [59]. This knowledge allowed us to employ fluorescently tagged annexins as highly specific Ca^{2+} sensors, rendering them a useful tool for the determination of $[Ca^{2+}]_i$ and, correspondingly, for establishing the outcome of a plasmalemmal injury even in heavily perforated cells [19].

Although all annexins associate with negatively charged phospholipids, it appears that several members prefer sites of distinct lipid composition. They can either bind to individual negatively charged phospholipids or to the assemblies of specific lipids such as lipid microdomains [27,57–59,78]. Annexin A1 is the only member of this protein family, which associates preferentially with ceramide-rich membranes under specific conditions – such as occurring during apoptosis or plasmalemmal injury – in which the ceramide concentration within the plasma membrane is elevated [27]. Provided that $[Ca^{2+}]_i$ is sufficiently elevated for annexin membrane binding, annexin A1 constitutes a reliable reporter for ceramide platforms allowing to evaluate the role of these specific lipid assemblies in apoptotic or injured cells.

Hence, annexin-based reporting is an important tool to predict cell fate or to determine the extent of physical membrane damage and the chances of cell survival. However, the annexins are not merely indicators of cell fate, but also take an active role in the repair process itself.

7. Annexins in plasma membrane repair

7.1. Resealing of mechanically induced lesions

Annexins are known to promote membrane aggregation and fusion and take part in exocytotic and endocytotic events [54]. It is this combination, together with their Ca^{2+}-dependency of membrane binding that makes them particularly well suited for a role in membrane repair. In this context it is interesting to note that early reports based on expression profiling and *in vitro* investigations suggested an association of annexins A1 and A2 with dysferlin in normal and in mechanically injured skeletal myotubes [79]. Both proteins were thought to mediate the exocytotic patch repair of skeletal muscle sarcolemma: after membrane injury annexins A1 and A2 were held to interact Ca^{2+}-dependently with intracellular vesicles. The authors suggested that the patch consisted of a chain of vesicles, interspersed with annexins A1 and A2 and anchored to the sarcolemma via a dysferlin link [79]. However, this model of membrane fusion events, which combines annexins and dysferlin still awaits experimental confirmation.

A requirement for annexin A1 in plasmalemmal repair was directly demonstrated in HeLa cells, following a laser-induced, localized plasmalemmal trauma. Annexin A1 translocated to the membrane and sealed the injured site in a Ca^{2+}-triggered fusion response [80]. The fusion could be specifically blocked by an inhibitory peptide, a specific antibody or a dominant-negative annexin A1 mutant protein incapable of Ca^{2+}-binding. These elegant experiments gave the first insight into the role of annexin A1 in cellular damage control.

7.2. Annexin-mediated microparticle release

Recent findings have implicated annexin A1 also in the resealing of plasmalemmal lesions induced by pore-forming toxins: down-regulation of annexin A1 expression levels with annexin A1-specific siRNA significantly decreased the capability of HEK 293 cells to withstand a SLO-attack [19]. The successful resealing of the SLO-induced lesions was accompanied by a massive shedding of plasmalemmal microparticles which contained no cytosol (Fig. 2) [19].

Plasma-derived, microparticles or microvesicles were originally branded as "platelet dust" for their procoagulant properties [81]. While the scientific literature around them has exploded, their classification remains a continual source of confusion: they have been variously catalogued according to size, intracellular origin, lipid composition, morphological characteristics or protein markers [82]. Recent reviews proposed their function to be determined by the cell of origin in combination with the mechanism of their release [82]. Flow cytometric methods have been instrumental in the isolation, purification and classification of microvesicles according to size or surface marker from the supernatants of live cells or from numerous body fluids: human plasma, serum, bronchial lavage, urine, amniotic fluid or milk [82]. Transported by the blood stream, some microparticles have been shown to carry bioactive cargo such as mRNA, microRNAs or proteins over long distances [83]. In keeping with their widely divergent origin, microparticles have been implicated in the conveyance of immune responses, tumour cell invasion, angiogenesis, bone mineralization or coagulation [82,84]. Their membrane orientation has been proposed to resemble that of the donor cell, hence they have been considered "miniature versions" of cells [82]. On the other hand, their pro-coagulant activities are linked to the exposure of phosphatidylserine on their outer surface [85]. This observations points to an "inside-out" or randomized orientation, i.e. loss of the plasmalemmal lipid asymmetry, of the microparticle membrane. At present the molecular mechanisms of microparticle shedding are unknown. In blood cells and platelets they are released in response to Ca^{2+} influx [86] and in erythrocytes they have been shown to contain annexin A7 [87].

Independent of the molecular details, the fusion of opposing plasma membrane segments is a prerequisite for microparticle formation, which are then pinched off and released into the extracellular space.

Annexin A1 is instrumental in the inward vesiculation in multivesicular endosomes – a process that is driven by a budding and fusion of the organelle-limiting membrane [65]. The formation of annexin A1 dimers or annexin A1/S100A11 heterotetramers is responsible for the membrane-fusion properties of this protein [88]. Our analysis confirmed that in cells, which were perforated by SLO, annexin A1 rapidly translocated to the "hot-spots" of Ca^{2+}-entry [19]. The affected, pore-containing region was then quarantined – presumably by annexin A1-mediated membrane fusion – budding outwards, and eliminated by shedding in form of extracellular microparticles (Fig. 2).

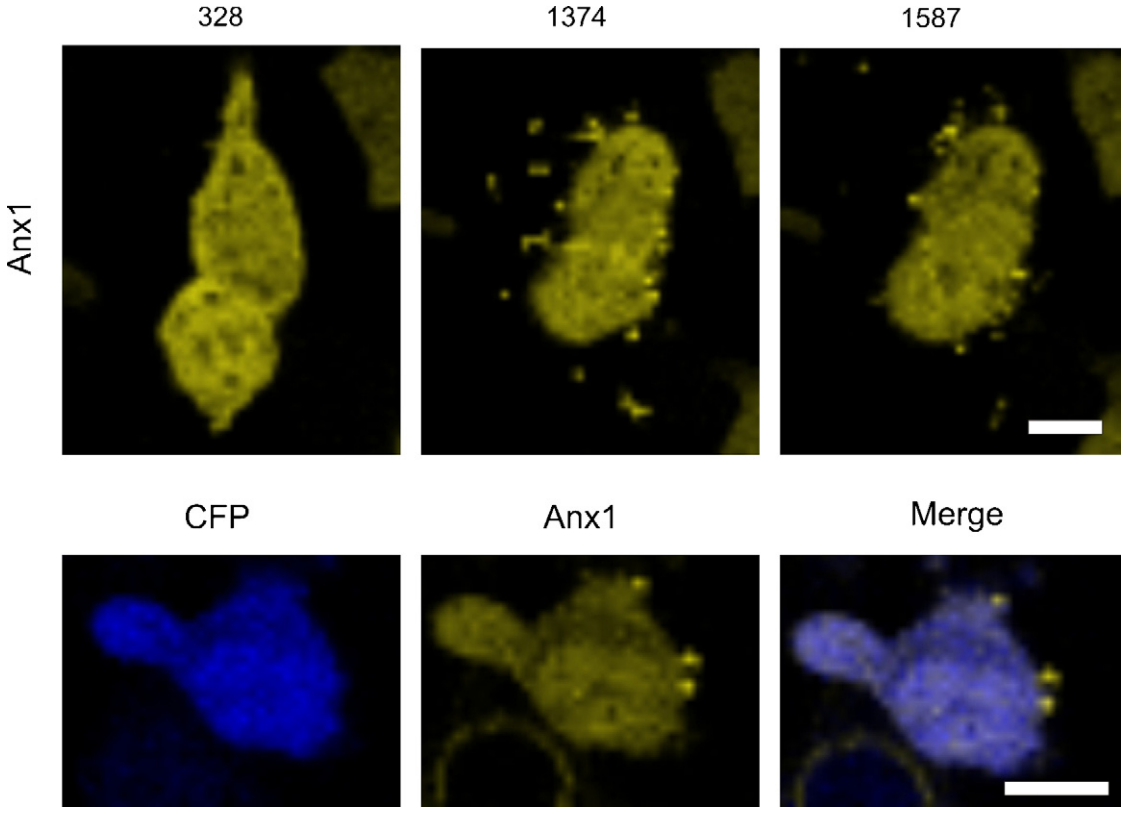

Fig. 2. Shedding of annexin A1-containing microparticles in SLO-permeabilized cells. Upper panel: SLO was added to cells, transfected with annexin A1 (Anx1-YFP, yellow). Between time points 1374 s and 1587 s after addition of SLO, the appearance of protrusions and the shedding of microparticles can be observed. Lower panel: SLO was added to cells double transfected with annexin A1 (Anx1, yellow) and cytoplasmic cyan fluorescent protein (CFP, blue). Note that the annexin A1-rich protrusions are devoid of CFP. Magnification bars = 10 μm. Reproduced from [19].

8. Blebbing: an annexin-mediated strategy to avoid Ca^{2+}-overload in extensively damaged cells

In injured cells the influx of extracellular Ca^{2+} triggers a process of plasma membrane blebbing [89]. Blebs are membrane protrusions, which emerge after the cortical actomyosin has contracted and become locally detached from the lipid bilayer [89]. The surge of cytoplasm through the cortical opening into the newly formed bleb is driven by hydrostatic pressure and leads to a herniation of the plasma membrane [89]. Therefore, blebbing can be intentionally elicited by a variety of Ca^{2+}-dependent or Ca^{2+}-independent mechanisms that activate myosin or induce a rearrangement of the subcortical cytoskeleton [89]. Blebbing occurs physiologically during cytokinesis, cell spreading and locomotion [89]. Yet, it is most often observed after cell injury and during apoptosis [89]. We have recently shown that cells in which blebbing was enhanced, were able to survive a Ca^{2+} overload better than cells in which blebbing was inhibited [90]. Thus we identified a hitherto unknown role of blebbing as a mechanism of resistance to plasmalemmal injury. In this capacity, blebs act as a trap for the damaged membrane segment and its adjoining cytosol, and are sealed off from the cell body by plugs of annexin A1 (Fig. 3) [90]. Compared with exocytosis, endocytosis or microparticle release, blebbing represents an indirect repair mechanism, which becomes operative after initial attempts at plasmalemmal resealing have failed. Blebbing by itself does not eliminate plasmalemmal lesions but instead creates an environment, which is favorable for membrane repair. It also allows the cell to resume tight control of its $[Ca^{2+}]_i$ in order to prevent cell death by an unrestrained influx of Ca^{2+}.

Blebbing might also be involved in protecting perforin-permeabilized cells from necrosis. Cytotoxic T-cells and natural killer cells utilize perforin, a pore-forming protein, to eliminate virus-infected cells and tumours. During perforin-induced cell death, the integrity of the plasma membrane is restored by repair responses that protect the cells from necrosis and permit granzymes to trigger apoptosis. Keefe et al. [91] have observed a selective accumulation of small extracellular dyes within the blebs of perforin-permeabilized cells that were undergoing apoptosis, which suggests that the plasmalemmal damage induced by perforin was confined within the blebs, whereas in irreversibly permeabilized, necrotic cells, the dyes were distributed throughout the entire cytoplasm.

Blebs have been observed in cells whose plasmalemma is intact and which are merely exposed to sub-lytic concentrations of pore-formers, various stress factors or undergo apoptosis [89]. These cells, in which the Ca^{2+}-homeostasis is usually compromised, succumb more readily to plasmalemmal injury than do healthy cells [19]. The sudden appearance of "multiple moth-eaten protrusions" following the exposure of cells to complement has been noted [16]. Blebs are known to appear even in the absence of extracellular Ca^{2+}, suggesting a Ca^{2+} release from intracellular stores in stressed cells [16]. It is presumably this mechanism which – in absence of a perforation – serves as advance warning of an imminent attack: random blebbing prepares individual semi-enclosed compartments, which would curtail the influx of Ca^{2+} into the cell body in the event of plasmalemmal perforation (Fig. 3).

9. Other annexins involved in plasmalemmal repair

Since most cells express several annexins with overlapping Ca^{2+}-sensitivities, it is likely that annexin-mediated membrane repair is a concerted action in which several members of the family take part. Indirect evidence for this fact is emerging from the plant field. There is broad consensus about the physiological role for

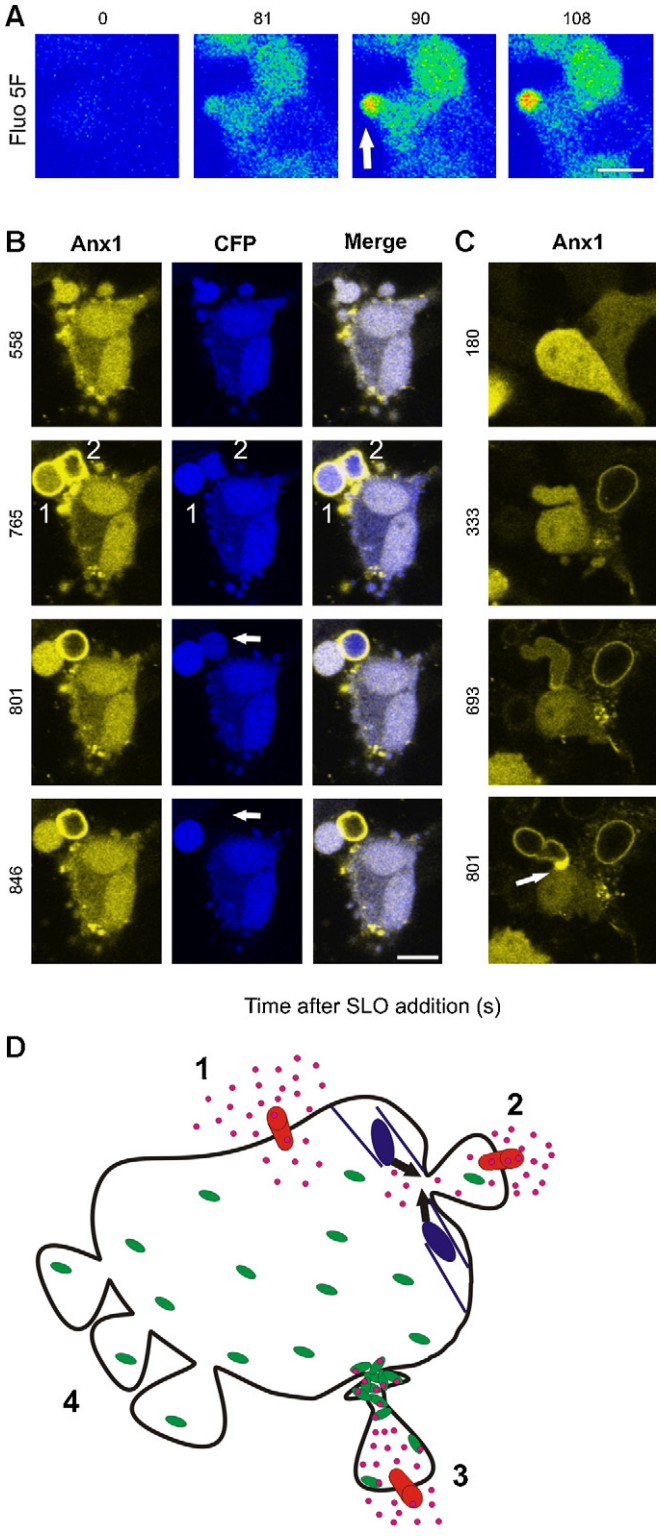

Fig. 3. Blebbing protects cells by isolating the damaged regions. (A) Intrableb elevation in Ca^{2+}. SLO was added (time = 0) to non-transfected HEK 293 cells that had been pre-loaded with Fluo 5F. At time points = 90 s and 108 s $[Ca^{2+}]$ was higher in the bleb (arrow) than in the cytoplasm. Fluorescence is shown in the rainbow mode: low $[Ca^{2+}]$ – blue; high $[Ca^{2+}]$ – red. (B) Blebbing, an elevation in $[Ca^{2+}]_i$ (membrane translocation of annexin A1) and the loss of cytoplasm (CFP-leakage) were simultaneously monitored in SLO-permeabilized HEK 293 cells and documented for two blebs. An efficient permeabilization of the cell was accompanied by a Ca^{2+} elevation in both of the blebs but not in the rest of the cytoplasm (time after SLO addition = 765 s). After permeabilization, the damaged plasma membrane of bleb 1 was efficiently repaired and excess of Ca^{2+} was pumped out, thereby resulting in a back translocation of annexin A1 (times after SLO addition = 801 and 846 s). In contrast, the repair mechanism in bleb 2 was inefficient: the intrableb $[Ca^{2+}]$ remained elevated and annexin A1 remained associated with the plasma membrane. The loss of cytoplasm was exclusively confined to the irreversibly perforated bleb (arrows): no leakage of cytoplasmic CFP occurred either from the repaired bleb or from the cell body. (C) Note a massive accumulation of annexin A1 at the base of the SLO-perforated bleb (arrow), which blocks the communication between the damaged bleb and the rest of the cytoplasm. The cell in the upper right-hand corner is irreversibly perforated. (D) An injury to the plasma membrane (1) disrupts the permeability barrier, thereby permitting an influx of Ca^{2+}. (2) The influx of Ca^{2+} triggers the myosin-driven contraction of the cortical cytoskeleton, instigating a localized increase in hydrostatic pressure within the surrounding cytoplasm: the plasma membrane around the lesion herniates to form a bleb containing the damaged membrane. If the cell is able to repair the membrane lesion, the elevated intra-bleb $[Ca^{2+}]$ is pumped out and eventually the bleb retracts. If resealing fails, intra-bleb $[Ca^{2+}]$ continues to rise. (3) Once $[Ca^{2+}]$ within the perforated bleb reaches 10 μM, intra-bleb annexin A1 binds to the plasma membrane and blocks communication between cell body and bleb. (4) Spontaneously protruding blebs. Plasmalemmal injury: injury – red cylinder; Ca^{2+} influx – pink circles. Injury-induced bleb formation: myosin-driven contraction – black arrows; cortical cytoskeleton – blue; cytoplasmic annexin A1 – green ovals. Plugged bleb: membrane-bound annexin A1 complexed with Ca^{2+} (green ovals pink circles). Magnification bars = 10 μm. Reproduced from [90].

various annexins in the development of plant stress tolerance [92]. However, it is interesting to note the difficulty in distinguishing the role of individual annexins in these stress responses [36], which suggests that several annexins might join forces.

Annexins A1 and A2 were proposed to cooperate with dysferlin in the Ca^{2+}-dependent sarcolemmal repair of skeletal muscle cells [79]. Our results likewise suggest that different annexins do collaborate in the resealing of the plasmalemmal lesions (Potez et al., unpublished). Highly Ca^{2+}-sensitive annexins are operative in the early stages of membrane injury, during which annexins with lower Ca^{2+}-sensitivity are ineffective. If partial depletion of a highly Ca^{2+}-sensitive annexin has occurred without plasmalemmal repair and $[Ca^{2+}]_i$ does not return to its homeostatic levels, annexins with a lower Ca^{2+}-sensitivity are employed as a second line of defence. Thus, the expression of a specific set of annexins might enable the cell to set its very own limit of Ca^{2+} tolerance.

10. Are annexins relevant for survival of multicellular organisms?

Experimental data firmly points to annexins as instruments for dealing with stress in cultured cells. Reports which reiterate this role in whole organisms are rapidly emerging. Of particular interest is data coming from the plant science field. Plant annexins originate from approximately 1000 million years ago and have developed into a particularly numerous and varied family [55]. A stress-induced upregulation of annexin gene expression has been observed, and studies monitoring abiotic stress responses in *Arabidopsis thaliana* suggest that a key function of plant annexins is the development of tolerance to drought- or oxidative stress [92,93].

The amount of data on annexins and on their potential role in various human diseases is staggering and the topic of several excellent reviews [54,94,95]. They are thought to be instrumental in the progression of cancer, diabetes and the autoimmune disorder anti-phospholipid syndrome [95]. Annexin A1 has been described as an important mediator of the anti-inflammatory effects of glucocorticoids [96]. Annexin A2 plays a role in vascular haemostasis by activating angiogenesis and by promoting fibrinolysis [97].

Here, we wish to concentrate on reports, in which a membrane repair function of annexins is observed *in vivo* and can be associated with the annexin-mediated repair mechanisms described above during experimental cell injury. Suggestive of a function in damage limitation are the observations that annexin A1

is released from inflamed colon in patients during bouts of ulcerative colitis [98], and annexin A1 is upregulated in the margin of healing gastric ulcers in mice [99]. Likewise concerned with wound healing is the observation that annexins A1, A2 and A5 in neurons and glia in rats are upregulated after experimental spinal cord trauma [100]. Increased vulnerability to a surfeit in Ca^{2+} has been observed in erythrocytes of annexin $A7^{-/-}$ mice, which proved to be resistant to malaria. Due to their decreased resistance to Ca^{2+} overload, *Plasmodium*-infected erythrocytes are unable to survive long enough for the parasite to reach maturity [101]. Well-documented is the upregulation of annexins A1 and A2 in patients diagnosed with limb-girdle muscular dystrophy 2B and Miyoshi myopathy [52]. These findings were interpreted as an attempt at membrane repair in the wake of a dysferlin deficiency. The extent of the annexin overexpression could be linked to the clinical severity of the case and was considered to be of prognostic significance [102]. We have shown that in skeletal muscle of patients undergoing a statin therapy for the treatment of hypercholesterinemia, there is evidence of an intracellular Ca^{2+} leak [103]. In skeletal muscle of these patients, the genes encoding annexins A1, A2, A4, A5 and A6 were found to be significantly upregulated [104]. This up-regulation which also included some of the annexins' S100 binding partners and dysferlin, indicates that Ca^{2+}-dependent, annexin-mediated membrane repair mechanisms are activated in the skeletal muscles of patients in whose organs cholesterol synthesis is significantly inhibited [104].

11. Pharmacological strategies directed towards membrane repair

In order to accelerate the recovery of damaged cells, pharmacological strategies must be directed either at the resealing of the plasmalemmal lesions with the help of artificial agents which mimic the properties of the lipid bilayer, or at the activation of the cell's own repair mechanisms.

Polyethylene glycol, a polar and hydrophilic molecule studied in membrane fusion [105] has shown considerable promise in the repair of mechanically lesioned neurons [106]. Another well-investigated agent is poloxamer 188 (P188). This substance is a high molecular weight triblock copolymer surfactant with both hydrophobic and hydrophilic domains. Both *in vitro* and *in vivo* studies have demonstrated the efficacy of P188 in the repair of damaged cell membranes [107]. P188 improves the structural and functional recovery of electropermeabilized skeletal muscle cells [108] and its chronic administration can prevent severe cardiac injury in dystrophic dogs [109].

Microparticles, released as a consequence of the plasmalemmal repair, are known to be pro-coagulant, pro-inflammatory and are supposed to play a role in cell proliferation or differentiation [110], however, reports are largely restricted to monitoring their number and cellular origin, and few attempts have been made to address the mechanism of their formation [110]. The mechanisms of generation or suppression of microparticle formation and their effect on the underlying pathology have recently been addressed in platelets. A monoclonal antibody blocking the interaction between von Willebrand factor and glykoprotein 1b α (GPIb α) significantly reduced microparticle formation and its prothrombotic effect on thrombin generation [111].

Blebbing is triggered by numerous agents which activate myosin and likewise, it is inhibited by substances, which block myosin activation [89,90]. Since until recently [90], an explanation into the physiological role of this phenomenon was lacking, so far no attempts have been made to promote blebbing by the application of pharmacologically active substances in selectively targeted cell populations, which are at risk of membrane damage.

Apart from annexin A5s use as an indicator of membrane injury, as yet no attempt has been made to pharmacologically manipulate and exploit the annexins' specific properties as plasma membrane rescue team.

12. Conclusion

Since membrane injury is a ubiquitous phenomenon, it is worth considering potential cellular alarm and defense mechanisms, aptly summarized as cellular non-immune defenses (CNIDs) by Aroian and van der Goot [112]. Here, we have outlined that cells have a multitude of options in order to meet attacks on the integrity of their plasmalemma.

Depending on the nature of their injury, cells can patch a mechanical lesion by exocytotic fusion of lysosomes, they can endocytose and thus internalize a protein-lined pore, or shed a small plasmalemmal lesion in the form of a microparticle. Moreover, with the formation of blebs, cells possess an early warning system, which enables them to foretell a potential attack. Depending on the mode of repair, they have the option – by the shedding of microparticles or the formation of blebs – to sacrifice a "limb" in order to save the body. Hovering between life or death by being suspended in a state of Ca^{2+} oscillations, represents another alternative in the cellular repertoire in dealing with membrane injuries, characterized by ongoing attempts at membrane repair.

Membrane repair mechanisms are mediated by several protein families. Whereas the SNAREs, the synaptotagmins and the ferlins are well-characterized in their functional capacity, we have here reviewed evidence for a role of the annexins in the management of plasmalemmal injury. The expression of several different annexins in any one cell, their staggered overlapping Ca^{2+}-sensitivities, their fusogenic properties and their distinct preferences for different lipid environments make them an ideal rescue team for emergencies such as an injury-induced deluge in intracellular Ca^{2+} and/or a change in the lipid composition of a cell membrane.

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