Cellular and Molecular Life Sciences

REVIEW

The annexins: spatial and temporal coordination of signaling events during cellular stress

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Abstract Annexins are a family of structurally related, Ca^{2+}-sensitive proteins that bind to negatively charged phospholipids and establish specific interactions with other lipids and lipid microdomains. They are present in all eukaryotic cells and share a common folding motif, the "annexin core", which incorporates Ca^{2+}- and membrane-binding sites. Annexins participate in a variety of intracellular processes, ranging from the regulation of membrane dynamics to cell migration, proliferation, and apoptosis. Here we focus on the role of annexins in cellular signaling during stress. A chronic stress response triggers the activation of different intracellular pathways, resulting in profound changes in Ca^{2+} and pH homeostasis and the production of lipid second messengers. We review the latest data on how these changes are sensed by the annexins, which have the ability to simultaneously interact with specific lipid and protein moieties at the plasma membrane, contributing to stress adaptation via regulation of various signaling pathways.

Keywords Annexin · Membrane binding · Calcium · pH · Signal transduction · Stress

Introduction

The plasma membrane is not just a barrier separating the cell from its environment, its proteins and lipids also coordinate and route the incoming and outgoing signals. The amplification and redistribution of signals are in many cases accomplished by a highly regulated intracellular release of Ca^{2+} or a controlled entry of Ca^{2+} through the plasma membrane. Chronic stress conditions are often accompanied by extracellular acidification, oxidative stress, cytokine-induced cell proliferation, and gene mutations [1]. There is a significant interplay between intracellular Ca^{2+} and pH homeostasis: changes in pH_i are often accompanied by an alteration of $[Ca^{2+}]_i$, intracellular acidification correlates with a marked increase in $[Ca^{2+}]_i$ due to the altered activity of calcium-entry channels, release from intracellular stores and its decreased affinity for Ca^{2+}-binding proteins [2, 3]. On the other hand, Ca^{2+} influx through store-operated channels mediates intracellular alkalinization [4], and the proteins involved in maintaining the pH homeostasis are regulated by Ca^{2+}-binding proteins, which are activated by many stimuli (Ras, PKC) sensitive to $[Ca^{2+}]_i$ [5]. Extracellular stress factors can incite contraction, division, secretion, cytokine-induced cell proliferation, gene mutations, or apoptosis. The basic stress signal can be as variable as the cellular response and is dependent on the cell type and the extent of the stimulus. Members of the annexin protein family are characterized by their distinct Ca^{2+}- and phospholipid-binding properties and have the ability to converge at the plasma membrane during stress. They act as intracellular sensors discriminating the incoming signals and thus may provide each cell type with individuality in its reaction to the changing environment.

Ca^{2+}-binding proteins: the annexin family

There are several classes of Ca^{2+}-binding proteins ranging from Ca^{2+}-gated channels within the membranes, proteins serving as Ca^{2+} buffers, and proteins that change their

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functions in response to Ca^{2+}-binding, thus directly participating in the signaling [6]. Based on the structure of their Ca^{2+}-binding sites, the Ca^{2+}-binding proteins can be classified as EF-hand proteins, C2-domain proteins and annexin, or type-II, Ca^{2+}-binding-site proteins. Annexins are a family of structurally related, Ca^{2+}-sensitive proteins that bind to negatively charged phospholipids [7] and establish specific interactions with other lipids and lipid microdomains [8, 9]. Annexins were initially discovered as proteins, mediating the Ca^{2+}-dependent aggregation of chromaffin granules [10]. Synexin, now known as human annexin A7, was the first member of the family whose Ca^{2+}- and membrane-binding properties were extensively studied [11]. Its discovery was followed by annexins A1 and A2 (then called lipocortin and calpactin), identified as substrates of Src and EGF protein kinases [12, 13]. Already the early studies identified a complex between annexin A2 and a member of EF-hand Ca^{2+}-binding S100 protein p11 (S100A10) [14]. The interaction of annexins with S100 proteins is a feature shared by most members of the family [15]. The 12 vertebrate annexins form the A family (annexins A1-A13, A12 left unassigned). The other annexins are currently classified into families B (invertebrates), C (fungi and unicellular eukaryotes), D (plants), and E (protists) [16]. The structure and diverse functions of annexins have been extensively reviewed [7, 17–19], so here we will only give a brief overview of the important features of these molecules, before concentrating specifically on their role in cell signaling events occurring at the plasma membrane during intracellular stress.

Molecular structure of annexins

All annexins possess a common folding motif, the C-terminal "core domain" [16]. The core domain incorporates functional sequences that are common for all members of the annexin protein family including Ca^{2+}-binding sites and sites responsible for the interactions with negatively charged phospholipids. In contrast, the NH_2-terminal domains are variable and are thought to confer specific properties of each individual annexin.

Crystal structures of annexins have been determined for the core domains and proteins with short NH_2-termini [20–22]. The Ca^{2+}-binding sites of all annexins are located within the conserved core domain, comprising four (eight in the case of annexin A6) annexin repeats, each 70 amino acids long, forming five α-helices connected by short loops. The annexin core has the shape of a slightly curved disc, with Ca^{2+}- and lipid-binding sites located at the convex face of the molecule. Ca^{2+}, which is essential for the membrane interaction of most annexins, simultaneously coordinates carbonyl and carboxyl groups of the protein

and phosphoryl groups at the glycerol backbone of membrane phospholipids [23]. Ca^{2+} binding leads to the conformational changes in the core domain, particularly in repeat III [8, 20, 24].

The variability of the NH_2-termini is thought to influence the specificity of the Ca^{2+}- and phospholipid-binding ability of the annexins. The NH_2-terminal domains of annexins harbor sites for post-translational modification as well as for the interaction with other proteins [17]. The EF-hand proteins of the S100 family form the largest group of annexin-associated proteins, which are bound to the NH_2-termini of annexins. The interaction of different annexins with S100 proteins has been the subject of two recent reviews [15, 25]. Here we discuss the annexin A2-S100A10 complex because its formation significantly alters the properties of annexin A2 and is implicated in many regulatory functions. Annexin A2 exists as a monomer (p36) or a heterotetramer (p90), in which two annexin molecules are associated with a dimer of S100A10 (p11). Annexin A2 forms a Ca^{2+}-independent complex with S100A10, whereas the interaction of other annexins with S100 proteins, e.g. the association of annexin A1 with S100A11, requires Ca^{2+}. Mutagenesis analysis showed that the NH_2-terminal domain of annexin A2 regulates Ca^{2+}-dependent membrane aggregation [26]. Due to the formation of a heterotetrameric complex with S100A10, annexin A2 gains the ability to aggregate membranes at micromolar Ca^{2+} concentrations [7].

The effects of the NH_2-termini on Ca^{2+} binding are modulated not only via their modifications such as phosphorylation or binding to other proteins, but also directly following the conformational changes in this region of the molecule [27]. The role of the NH_2-terminus in the modulation of Ca^{2+} sensitivity of lipid binding is apparent in the case of annexin A1. Truncated annexin A1 has a typical core structure [28], resembling annexin A5 and the core domain of annexin A2. The NH_2-terminus of the full-length protein inserts into the core, replacing and unwinding one of the helices. Upon Ca^{2+} and membrane binding, the NH_2-terminus is expelled from the molecule and becomes available for other interactions, and the core Ca^{2+} binding site is restored [29]. It is possible that in the absence of Ca^{2+} ions, annexin A1 might be in its inactive form, and only after Ca^{2+} and/or phospholipid-binding, which promote an overall conformational change, annexin A1 assumes a structure allowing it to aggregate membranes.

Some annexins, including annexin A1, A2, A4, and A7 can promote membrane interaction in vitro, which has led to the hypothesis of their involvement in endocytosis and exocytosis. The mechanism of simultaneous interactions with two membranes could be either via protein-protein interactions, such as the one described for the A2-S100A10

heterotetramer [30], or directly via two independent membrane-binding sites within one annexin molecule [31, 32]. Alternatively, since annexin A1 binds S100A11 protein in a Ca^{2+}-dependent manner, the complex might form a symmetrical membrane-binding unit, linking two membranes [33]. Annexin A6 contains two cores, which are flexible and can orient themselves, allowing the molecule to interact with one or two membranes [34].

Expression and distribution of annexins

The expression of multiple annexins within each cell and the high similarity of the annexin molecular structure indicate the importance of these proteins. Indeed, although the distribution of annexins varies in human tissues and organs, these proteins are highly abundant in smooth muscle (annexins A1, A2, A4, A6) [8, 35], cardiomyocytes (annexins A4, A5, A6, and A7) [36], and endothelial cells (annexins A1, A2, A5, and A6) [37]. On the other hand, expression of some annexins is restricted to specific tissue types: annexin A3 to neutrophils and placenta, annexin A8 to skin, annexin A9 to tongue, and annexin A13 to small intestine [38–40].

When cellular functions deteriorate, frequent changes in the expression levels of annexins are observed [41]. Notably, most forms of human cancer are associated with significant alterations in the expression pattern of annexins, consistent with the role of these proteins in linking plasma membrane to cytoskeleton, maintenance of intracellular contacts, and endocytosis [42]. Annexins A1, A2, A4, and A6 are down-regulated in prostate cancer [43], and annexin A7 is a candidate tumor suppressor gene in prostate cancer, inhibiting prostate cell migration [44]. In acontractile bladders, concomitant with degenerative changes in smooth muscle cells, the expression of annexin A6 is reduced, which might have a bearing on the fine-tuning of the contractile apparatus [45].

At low $[Ca^{2+}]_i$, annexins are diffusely distributed throughout the cytosol. After stimulation and $[Ca^{2+}]_i$ elevation, they translocate to cellular membranes [35] (Fig. 1). Annexin A2 is involved in intracellular vesicle movement [46] and is associated with endosomal function. Endosomal localization has also been reported for annexins A1 and A6 [47, 48]. Annexin A5 has previously been associated with late endosomes [49, 50]. Annexins A5 and A6 have been associated with the Golgi apparatus, vacuolar membranes, and the endoplasmic reticulum [50, 51]. Several annexins (among them annexins A1 and A5) have been localized to the nucleus [52]. Hence, the intracellular translocation of annexins represents an important mechanism for targeting specific substrates and for the control of individual protein or lipid function.
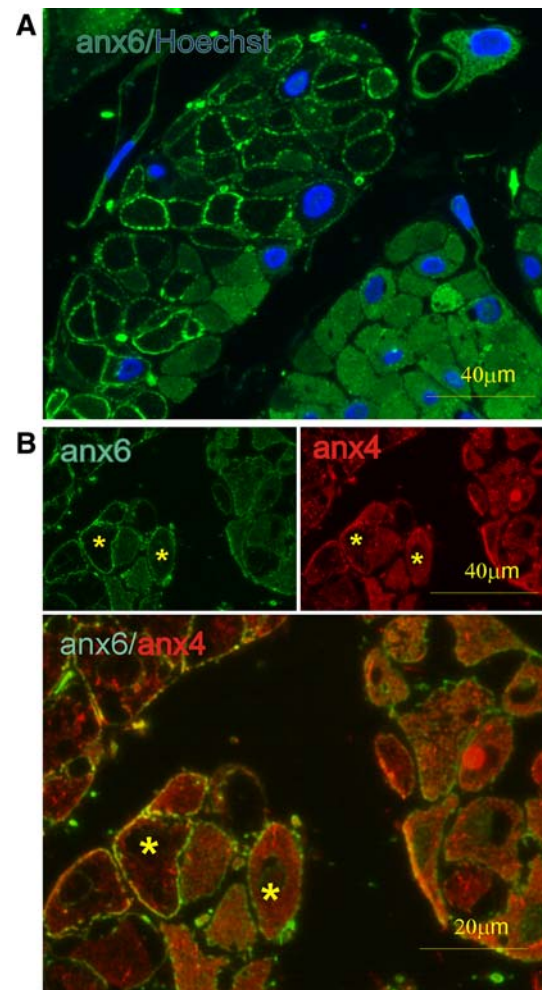

Fig. 1 Ca^{2+}-dependent localization of annexins A4 and A6 in smooth muscle cells. Fluorescent micrographs of transverse ultra-thin cryosections through human myometrium. **a** Double labeling with a monoclonal antibody against annexin A6 (anx6: *green*) and nuclear dye Hoechst (Hoechst: *blue*). Contracted cells are outlined by annexin A6 translocation to the plasma membrane, whereas in relaxed cells, this protein shows a diffuse cytoplasmic distribution. **b** Double labeling with a polyclonal antibody against annexin A6 (anx6: *green*) and a monoclonal one against annexin A4 (anx4: *red*). Note the cytoplasmic localization of annexin A4 in contrast to plasma membrane-associated annexin A6 (*asterisks*), indicating that in some cells, $[Ca^{2+}]_i$ is above annexin A6's, but below annexin A4's sensitivity for membrane translocation

Annexins regulate plasma membrane architecture

Annexins interact with specific lipids

Annexins are defined through their ability to bind negatively charged phospholipids, however, there are differences in binding to phospholipids with particular headgroups [phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidic acid (PA)] [53]. Negatively charged phospholipids best promote binding of most annexins to

phosphatidylcholine (PC)-containing membranes [54]. A comparison of 11 different types of lipids revealed that other factors such as the type of ion contributing the charge and head-group size were also important. Mixing phosphatidylserine and phosphatidylethanolamine or phosphatidylserine and phosphatidylinositol in the same PC vesicle synergistically enhanced annexin A4-membrane binding [54].

Lipid A is an active component of bacterial endotoxin in the bacterial envelope [55]. Annexins A1 and A2 were found to bind to this lipid, and the binding might have a role in suppressing the cellular and systemic responses to endotoxin by inhibiting its interactions with cellular receptors or accessory binding proteins [55].

Phosphatidylinositol 4,5-bisphosphate ($PtdIns_{4,5}P_2$ or PIP_2) is a major phosphoinositide of the plasma membrane that comprises about 1% of the plasma membrane phospholipids [56]. $PtdIns_{4,5}P_2$ is the precursor of the second messengers inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) [57] and plays a role in regulation of actin cytoskeleton [58]. $PtdIns_{4,5}P_2$ is a binding partner of annexin A2 at the sites of its membrane-dependent interaction with actin [59, 60]. Direct binding of annexin A2 to $PtdIns_{4,5}P_2$ in actin-rearrangement sites was demonstrated by Rescher et al. [60], who observed the accumulation of annexin A2 at the attachment sites of enteropathogenic *E. coli*, together with a concentration of cholesterol and an aggregation of GPI-anchored proteins [60, 61]. Simultaneously, in liposome sedimentation assays, Hayes et al. [59] demonstrated that recombinant annexin A2 bound to $PtdIns_{4,5}P_2$ but not to other poly- and mono-phosphoinositides in the presence and absence of Ca^{2+}. Since $PtdIns_{4,5}P_2$ is a precursor for many lipid second messengers (IP_3 and DAG) and involved in vesicular trafficking and cell motility, these results support the role of annexin A2 in the regulation of membrane-cytoskeleton dynamics in vesicle trafficking and indicate a possibility of its involvement in other cell signaling events [59].

Cholesterol is an important membrane constituent, therefore the association of annexins with cholesterol and cholesterol-rich membranes was extensively investigated. Annexin A2 was shown to interact with cholesterol and its distribution was modulated by the subcellular distribution of cholesterol in cells from patients with the cholesterol-storage disorder Niemann-Pick C [47]. The authors conclude that annexin A2 forms cholesterol-containing platforms on early endosomal membranes and that these platforms regulate the onset of the degradation pathway in animal cells [47]. Annexin A2 was shown to interact with endosomal membranes independent of Ca^{2+} [62], and this binding was demonstrated to be mediated by cholesterol

[9, 63]. Annexin A2 is tightly membrane-associated in a cholesterol-dependent manner and interacts physically with elements of the cortical actin cytoskeleton. Taking into account these observations, the authors proposed that this protein serves as an interface between membranes containing high amounts of cholesterol and the actin cytoskeleton. In addition, annexin A2 is thought to play a role in cholesterol uptake: it associates with caveolin-1 in cultured cells during cholesterol uptake [64] and forms a tight complex with caveolin in zebrafish intestinal epithelium [65]. Annexin A2 plays an important role in exocytosis in chromaffin cells [66], where its function is mediated by cholesterol- and sphingomyelin-rich lipid microdomains (lipid rafts): annexin A2 influenced exocytosis via formation or stabilization of lipid rafts, and reduction of annexin A2 reduced exocytosis at the stage before membrane fusion [67]. The annexin A2-S100A10 complex is involved in the formation of E-cadherin-based adherens junctions, in a cholesterol-dependent manner [68]. However, biochemical data indicate that annexin A2 does not bind cholesterol directly, and these effects are strictly dependent on the presence of PS in liposomes [69]. Cholesterol in liposomes is able to increase Ca^{2+}-dependent binding of NH_2-terminally truncated annexins, showing that the NH_2-terminus is not involved. Cholesterol increases the Ca^{2+} sensitivity of membrane binding of annexins A2 and A6, possibly by clustering/stabilizing PS-rich domains.

Similarly, annexins A5 and A6 show a cholesterol-mediated enhancement of their Ca^{2+}-dependent binding to membranes, indicating that the annexin core domain is responsible for the cholesterol-mediated effects [70]. Annexin A6 is involved in the endocytosis of low-density lipoprotein (LDL) by regulating the entry of ligands into the pre-lysosomal compartment [71]. Cholesterol was shown to influence the membrane-binding affinity and intracellular distribution of annexin A6, and changes in its intracellular distribution and concentration in different subcellular compartments caused the reorganization of intracellular pools of annexin A6 [72]. Annexin A6 plays an important role in the transport of both cholesterol and caveolin. Cells expressing high levels of annexin A6 are characterized by an accumulation of caveolin-1 in the Golgi complex [73]. This was associated with a sequestration of cholesterol in the late endosomes and lower levels of cholesterol in the Golgi and the plasma membrane, both likely contributing to retention of caveolin in the Golgi apparatus and reduced number of caveolae at the cell surface. The elevated expression levels of annexin A6 perturb the intracellular distribution of cholesterol, indirectly inhibiting the exit of caveolin from the Golgi complex.

Annexins and membrane rafts

Compartmentalization of the plasma membrane has been shown to be a prerequisite for the efficient and precise processing of signals across the plasma membrane. Lateral segregation of cholesterol- and sphingomyelin-rich lipid microdomains (rafts) and glycerophospholipid-containing non-raft regions has been proposed to play a role in a variety of biological processes [74–76]. The early studies indicated a Ca^{2+}-dependent association of certain annexins with lipid rafts [77, 78]. In mammary epithelial cells, CD44, the major cell surface receptor for hyaluronic acid, was shown to localize to detergent-resistant cholesterol-rich raft microdomains that contain annexin A2 at their cytoplasmic face. These CD44-containing lipid microdomains also interact with the underlying actin cytoskeleton [78]. When examining the structure of smooth-muscle plasmalemma, we showed that annexins A2 and A6 translocate to the detergent-resistant sites of the smooth-muscle cell in a Ca^{2+}-dependent manner [8]. We further extended these observations by showing that the members of the annexin protein family each associate with different membrane compartments [79] (Fig. 2). Selective extraction of cholesterol prevents the relocation of annexin A6, but not of annexin A4 to the sarcolemma [35]. In contrast, the extraction of glycerophospholipids results in solubilization of annexins A1 and A4 whereas annexins A2 and A6 remain associated with the detergent-resistant membranes [35].

Upon binding, some members of the family have been shown to induce segregation of membrane lipids, with certain phospholipids accumulating underneath the sites of annexin attachment [80]. By means of scanning force and fluorescence microscopy of artificial membranes
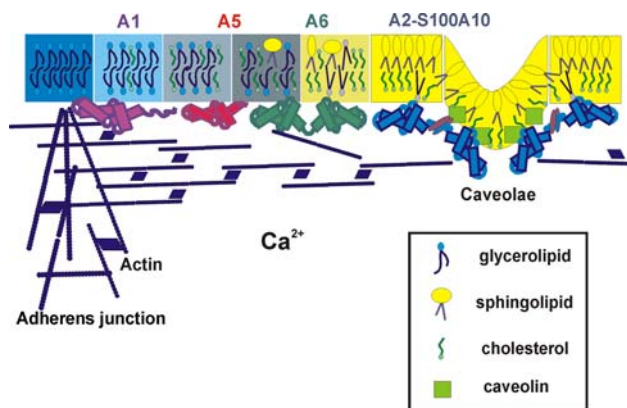

Fig. 2 Annexins interact with different lipid microdomains. Interdependent but not identical gradients of individual lipids exist within the membrane bilayer. The lipid moieties are responsible for targeting annexins to their respective places within the membrane. The segregation is further stabilized via an interaction of the membrane with the submembraneous cytoskeleton

immobilized on mica surfaces, the lateral organization of the annexin A2-S100A10 heterotetramer and its influence on the lateral organization of the lipids within the membrane have been elucidated. A depletion of the 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine domains was observed in the vicinity of annexin A2 tetramer protein domains, indicating that it was a peripheral membrane-binding complex capable of inducing lipid segregation [81]. Additionally, in the presence of Ca^{2+}, annexin A2 caused the association of rafts [8]. It is therefore possible that the annexins might constitute a means of regulating Ca^{2+}-dependent raft-assemblage [8]. In smooth-muscle cells, the raft binding of annexin A2 could be interrupted following the proteolytic cleavage of the annexin [82]. This cleavage is initiated by calpain and is dependent on $[Ca^{2+}]_i$ and the presence of an intact contractile apparatus. Proteolysis abolishes annexin-binding to the plasma membrane and might result in the rearrangement of membrane constituents followed by the interruption of segregation-dependent signaling events [85].

The important function of annexins in raft organization was further strengthened by our experimental data showing that the structural changes in plasma membrane of contractile cells during development were accompanied by alterations of expression and localization of specific annexins [83]. The differences in skeletal and smooth-muscle sarcolemma can already be observed at the microdomain level and are characterized by a relative paucity of lipid rafts in differentiated skeletal muscle. However, the undifferentiated skeletal myoblasts and myotubes closely resemble smooth-muscle cells in their expression of raft-associated lipids and proteins. During differentiation in the early perinatal period, the plasma membrane of skeletal muscle undergoes a striking reorganization of its lipid and protein components; we showed that 5′-nucleotidase activity markedly decreased, concomitantly with transverse tubular invagination and terminal maturation of the skeletal-muscle sarcolemma. Simultaneously, membrane microdomain segregation became less apparent and raft-associated annexins A2 and A6 were downregulated. Annexin intracellular localization was also changing; the annexins were diffusely distributed within myotubes, transiently co-localized with caveolin-3 during embryogenesis, and expressed in the T-tubules within mature myocytes.

During monolayer culturing, smooth-muscle and endothelial cells are known to undergo dramatic cytoskeletal and plasma-membrane rearrangements. This extensive reorganization of the plasma membrane coincides with changes in the expression levels of the lipid raft marker proteins annexins A2, A6, and 5′-nucleotidase [84]. These three raft markers were massively upregulated in monolayer cultures of endothelial or smooth-muscle cells

(single or co-cultures) compared with the levels in native smooth-muscle tissue or three-dimensional spheroids. Similarly, in the smooth-muscle cultures, the transition from normal spindle-shaped to epithelioid morphology is accompanied by structural changes in sarcolemma: the epithelioid cells had 30% higher levels of cholesterol and correspondingly lower levels of glycerophospholipids [85]. These changes in sarcolemmal lipid composition were associated with a reduced diversity in the range of membrane-binding annexins: expression of annexins A1, A4, A6, and A7 was lost, whereas the levels of annexins A2 and A5 were increased. Interestingly, in epithelioid cells, cholesterol was more susceptible to extraction, implicating loss of annexins and altered intracellular distribution of caveolin in membrane stabilization.

Annexin A1 interacts with ceramide platforms

The ability of annexins to sense Ca^{2+} signals and specific lipid second messengers is further elucidated in our recent study, which demonstrates the involvement of annexin A1 in the Ca^{2+}-dependent production of ceramide and its clustering into platforms at the plasma membrane of living cells [86]. Ceramide is a key lipid mediator in cellular processes such as differentiation, proliferation, growth arrest, and apoptosis [87, 88]. Ceramide is synthesized de novo in the ER and transported to the Golgi apparatus where it is converted into complex sphingolipids. Alternatively, intracellular sphingomyelinases, triggered by a variety of pro-apoptotic signals, can convert sphingomyelin to ceramide at the plasma membrane [89]. Upon its genesis during apoptosis, ceramide self-associates into platforms, thereby promoting extensive structural changes within the plasma membrane [90, 91]. Hence, its classical role as a second messenger is being challenged in favor of a more general one, involving gross reorganization of membrane structure, clustering of signaling molecules, and an amplification of vesicle formation, fusion, and trafficking.

We showed that ceramide increased the affinity of annexin A1-membrane interaction [86]. In the physiologically relevant range of Ca^{2+} concentrations, this leads to an increase in the Ca^{2+}-sensitivity of annexin A1-membrane interaction. Among proteins of the annexin family, the interaction with ceramide platforms is restricted to annexin A1 and is conveyed by its unique NH$_2$-terminal domain (Fig. 3). Using fluorescently tagged annexin A1 as a reporter for ceramide platforms and annexin A6 as a non-selective membrane marker, we visualized ceramide platforms and provided evidence for a ceramide-driven segregation and internalization of membrane-associated proteins (Fig. 4).

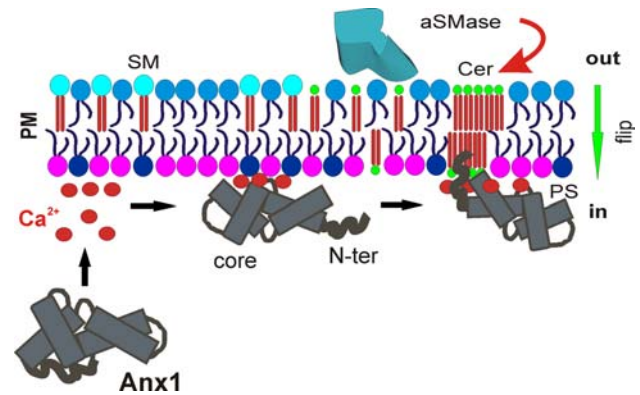Among other functions, annexin A1 is believed to act as a mediator of anti-inflammatory glucocorticoids [92].

Fig. 3 The interaction between annexin A1 and ceramide platforms. Under conditions of stress, accompanied by sustained elevation of $[Ca^{2+}]_i$, acid sphingomyelinase (aSMase) converts sphingomyelin (SM) to ceramide (Cer) (*red arrow*). The *green arrow* indicates the spontaneous transbilayer equilibration of ceramide in the plasma membrane (PM) between the extra- and intracellular compartments (*out*, *in*). The Ca^{2+}-mediated interaction between the annexin core and anionic phospholipids induces conformational changes in the annexin A1 molecule that result in the previously buried NH$_2$-terminal domain becoming externally accessible. The unique NH$_2$-terminus of annexin A1 conveys the selective interaction of the protein with the phase boundaries and its penetration into the highly ordered domains of tightly packed acyl chains formed in the membrane upon generation and self-association of ceramide. *Anx1* Annexin A1, *core* annexin A1 core domain, *N-ter* unique NH$_2$-terminal domain of annexin A1

Annexin A1$^{-/-}$ cell lines overexpress Cox-2 and cPLA2 and are insensitive to dexamethasone, implicating annexin A1 in the inhibition of inflammatory and proliferative signal transduction pathways [93]. Additionally, this protein plays a role in the execution of apoptosis [94] and the clearance of apoptotic cells by macrophages [95]. An interaction between annexin A1 and ceramide platforms occurs under conditions of cellular stress. Annexin A1 can influence the dynamic of lipid condensed domains in phospholipid monolayers [96]. This suggests that, in living cells, the formation of ceramide platforms might be promoted by annexin A1 binding. Annexin A1 has been shown to be released by apoptotic cells [95]. Our results indicate that the formation of ceramide platforms within the outer lipid bilayer together with the exposure of phosphatidylserine might serve as a stimulus for annexin A1 binding to apoptotic cells and mediate an annexin A1-dependent clearance of apoptotic cells by macrophages and annexin A1-induced apoptosis of surrounding cells [94].

Membrane curvature is sensed by annexin B12

Changes in membrane curvature take place during many cellular processes, including movement, division, and vesicular trafficking. Curvature can be dynamically modulated by changes in lipid composition, the oligomerization

Fig. 4 Binding of annexin A1 to ceramide platforms under conditions of cellular stress. The formation of annexin A1-positive ceramide platforms was monitored in live Jurkat T-cells co-transfected with annexin A1-YFP (Anx1: *yellow*) and annexin A6-CFP (Anx6: *blue*). The cells were maintained in Tyrode's buffer containing 2 mM $CaCl_2$ and challenged with Ca^{2+}/ionomycin at 30 s. Data sets were obtained with a ZEISS LSM 510 Meta at the time points indicated. Sustained elevation of $[Ca^{2+}]_i$ results in the formation of ceramide, which accumulates within plasma membrane platforms (*arrows*). Annexin A1, but not annexin A6, associates with ceramide platforms
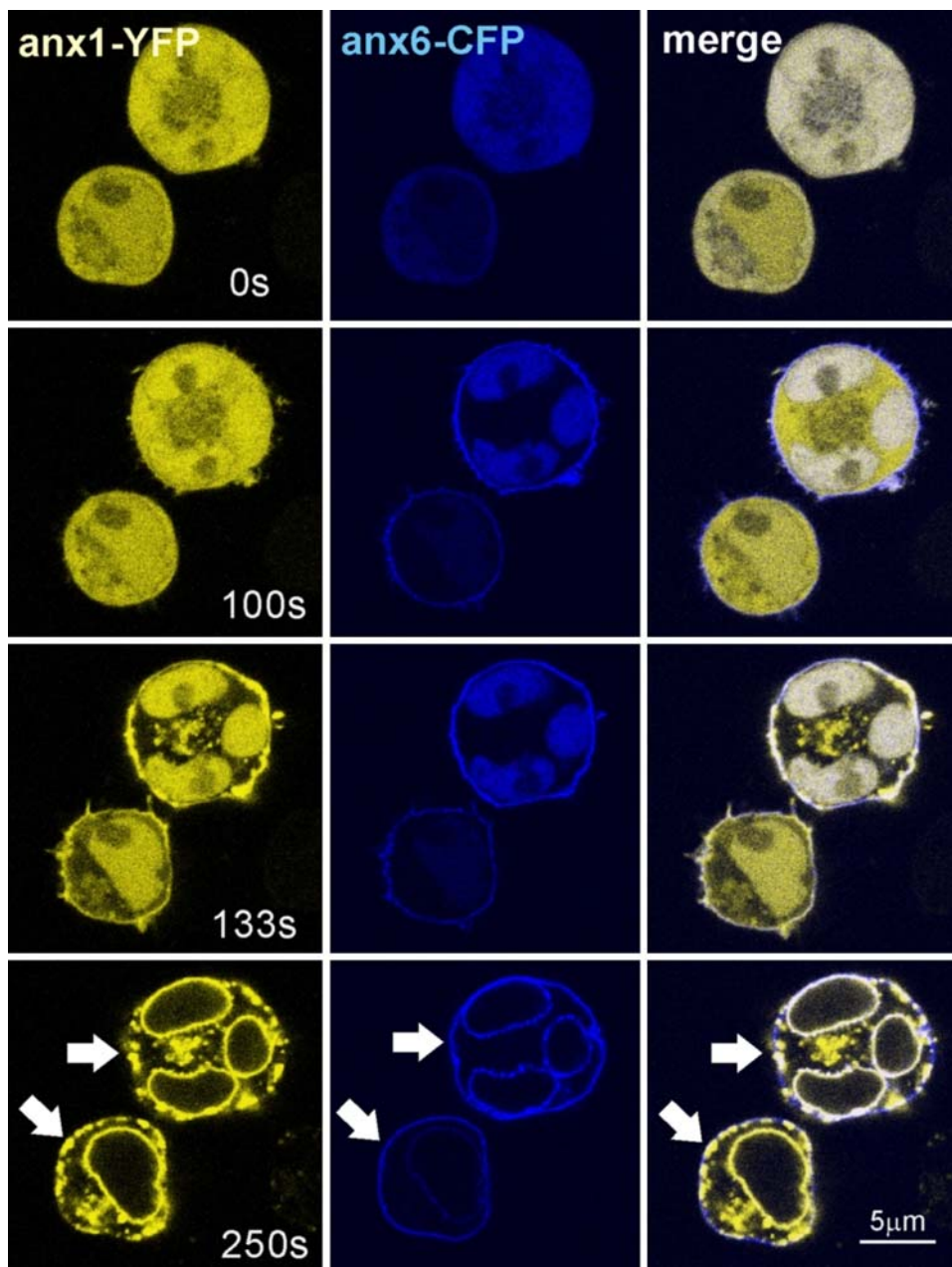
of curvature scaffolding proteins, and the reversible insertion of protein regions that act like wedges in membranes. There is an interplay between curvature-generating and curvature-sensing proteins during vesicle budding [97]. For example, the Golgi-associated protein ArfGAP1 has an unusual membrane-adsorbing amphipathic alpha-helix: its polar face is weakly charged, containing mainly serine and threonine residues, and it was shown that this feature explained the specificity of ArfGAP1 for curved versus flat lipid membranes [98].

In a recent study by Fischer et al. [99], a combination of biochemical and biophysical approaches was used to analyze the interaction of annexin B12 with membranes of different curvatures. The authors observed a selective and Ca^{2+}-independent binding of annexin B12 to negatively charged vesicles that were either highly curved or contained lipids with a negative intrinsic curvature. This novel curvature-dependent membrane interaction induced major structural rearrangements in the annexin protein and resulted in a backbone fold that was different from that of the well characterized Ca^{2+}-dependent membrane-bound form of annexin B12. Following curvature-dependent membrane interaction, the protein retained a predominantly alpha-helical structure but underwent inside-out refolding that brought previously buried hydrophobic residues into contact with the membrane. These structural changes were

reminiscent of those previously observed following a Ca^{2+}-independent interaction of annexins with membranes at mildly acidic pH (see below), yet they occurred at neutral pH in the presence of curved membranes. Therefore, annexin B12 could be a sensor of membrane curvature, thus explaining the previously reported Ca^{2+}-independent membrane interaction of annexins in vivo occurring at neutral pH.

Many signaling proteins are modulated by changes in lipid composition and concomitant alterations of the curvature. The activity of membrane-associated PKC was shown to be tightly controlled by the physical properties of the membrane lipid bilayer, in particular, curvature stress, which is induced by bilayer-destabilizing lipid components [100, 101]. It is therefore possible that the ability of annexins to sense lipid-induced changes in membrane curvature could bring them into contact with other signaling molecules at the plasma membrane, i.e. PKC, and thus allow them to modulate different signaling pathways both Ca^{2+}-dependently and Ca^{2+}-independently.

Annexins and the cytoskeleton

The cortical cytoskeleton has an important function in stabilizing the plasma membrane and is involved in the regulation of multiple cellular processes under normal conditions and during stress. Several members of the annexin family are capable of binding to actin [102]. Annexin A2 binds F-actin [103], and its knock-down decreases the migration of human glioma cells [104]. Annexin A2 is essential for actin-dependent vesicle transport, as discussed above [59, 105]. In spontaneously motile cells, Hayes et al. [106] demonstrated that annexin A2 was observed in dynamic actin-rich protrusions, and its depletion led to the accumulation of stress fibers and loss of protrusive and retractile activity. Annexin A2 reduced the polymerization rate of actin monomers in a dose-dependent manner by inhibiting the filament elongation at the barbed ends. These results point to the essential role of annexin A2 in the plasticity of a dynamic, membrane-associated actin cytoskeleton, achieved through direct interactions with polymerized and monomeric actin [106]. In polarized epithelial cells, annexin A2 in complex with S100A10 and AHNAK protein recruit the cortical actin cytoskeleton and have been implicated in the regulation of the actin cytoskeleton organization at the lateral plasma membrane [107].

Recently, it has been shown that leukemia-cell-associated annexin A8 binds Ca^{2+}-dependently and with high specificity to $PtdIns_{4,5}P_2$, interacts with F-actin, and associates with late endosomes [108, 109]. Interestingly, these properties of annexin A8 closely resemble annexin A2, which could be an indication of the functional redundancy within the annexin protein family.

The role of annexin A6 in endocytosis has been linked to cytoskeletal modification [110]. It is required for the budding of clathrin-coated pits from human fibroblast plasma membranes: annexin A6 binds to β-spectrin at the cell surface, consequently recruiting and activating a calpain-like protease. This seems to open the actin cytoskeleton to facilitate the initial steps of endocytosis. In smooth-muscle cells, annexin A6 together with annexin A2 forms a Ca^{2+}-dependent, plasmalemmal complex with actin filaments [111]. These annexins can also associate with F-actin-containing stress fibers in cultured smooth-muscle cells or fibroblasts in a Ca^{2+}-independent manner [112]. A complex of annexin A6 with actin has been suggested to stabilize the cardiomyocyte sarcolemma during cell stimulation [113].

In activated platelets, in high $[Ca^{2+}]$, annexin A5 was shown to associate with the cortical cytoskeleton by forming a specific bond to γ-actin [114]. Similarly, annexin A1 has been identified as a Ca^{2+}- and F-actin-binding protein in fractionated brain cortex [115]. In addition, annexin A1 interacts with profilin [116]. The interaction between annexin A1 and profilin was implicated in the regulation of the dynamic membrane-cytoskeleton organization, based on the submembrane localization of annexin A1 and the regulatory activity of profilin on the cytoskeleton [117]. During the onset of diabetes, the endothelial plasma membrane proteins actin, annexin A1, annexin A2, the p34 subunit of the Arp2/3 complex, and the Ras suppressor protein-1 become glycated [118]. The authors suggest that the nonenzymatic glycosylation of the cortical cytoskeleton-associated proteins has possible consequences for the fluidity of the endothelial plasma membrane and impairment of the endothelial mechanotransducing ability during hyperglycemia.

Annexins require different $[Ca^{2+}]_i$ for membrane interaction

Earlier studies using synthetic lipid vesicles [53, 54], chromaffin granules [26], and neutrophil secretory vesicles [119] demonstrated that annexins have diverse Ca^{2+} sensitivities of lipid binding. The individual $[Ca^{2+}]$ required for this association differ greatly, depending on the experimental setup and the source of the protein, which complicates a comparison of the data. In addition, the lipid composition of the bilayer is known to influence the affinity of annexins to the synthetic membranes [120].

Smooth-muscle-cell contraction is regulated by changes in $[Ca^{2+}]_i$. The smooth muscle expresses a broad array of annexins [111]. Using biochemical methods, we showed that each annexin required a subtly different individual level of free calcium for its translocation to the plasma membrane [35]: around 300 nM for annexin A2, 700 nM

for annexin A6, 1 μM for annexin A4, and 1.5 μM for annexin A1. Similarly, when examining the Ca^{2+}-induced translocations of four members of the annexin family and of two truncated annexins in live cells, we demonstrated that these proteins interact with the plasma membrane as well as with internal membrane systems in a highly coordinated manner [121]. In cells stimulated with ATP or carbachol, annexin A2 briefly associates with the plasma membrane during the transient elevation in $[Ca^{2+}]_i$ corresponding to a Ca^{2+} release from intracellular stores (Fig. 5a). Simultaneous Ca^{2+} imaging in transfected cells showed that the timing and duration of this association exactly corresponds to the $[Ca^{2+}]_i$ elevation. Annexin A6 translocated to the plasma membrane only when a sustained Ca^{2+} influx was induced by activation of store-operated channels with thapsigargin (Fig. 5b). A similar behavior has been observed for PKCα in differentiated PC12 cells following stimulation of ionotropic P2X receptors [122]. Hence, apart from the amplitude, the duration and/or the site of the influx (close to the plasma membrane) influenced the translocation of annexin A6. Annexin A2 is the most Ca^{2+}-sensitive of the studied proteins, followed by annexin A6, annexin A4, and annexin A1 (Fig. 5c). The calcium sensitivity of annexin A2 increases further following association with S100A10 [121]. Upon elevation of $[Ca^{2+}]_i$, annexins A2 and A6 translocate to the plasma membrane, whereas annexins A4 and A1 also become associated with intracellular membranes and the nuclear envelope. The NH_2-terminus has a modulatory effect on plasma membrane binding: its truncation increases the Ca^{2+} sensitivity of annexin A1 and decreases that of annexin A2 [121].

A study carried out using fluorescent protein-tagged annexins in neuroblastoma cells resulted in essentially the same order of Ca^{2+} sensitivity [123]. The FRET experiments revealed that most annexins were likely to occupy particular membrane regions, with very little lateral mobility. Given the fact that several annexins are present within any one cell, it is likely that they form a sophisticated $[Ca^{2+}]$ sensing system, with a regulatory influence on other signaling pathways.

pH-dependent membrane interaction and channel activity of annexins

Apart from the typical Ca^{2+}-dependent membrane binding described above, annexins have the ability to associate with both synthetic and natural membranes in the absence of Ca^{2+} [124–126], which points to the existence of pH-dependent binding mechanisms, most likely mediated by hydrophobic interactions. Isas et al. [127] reported that
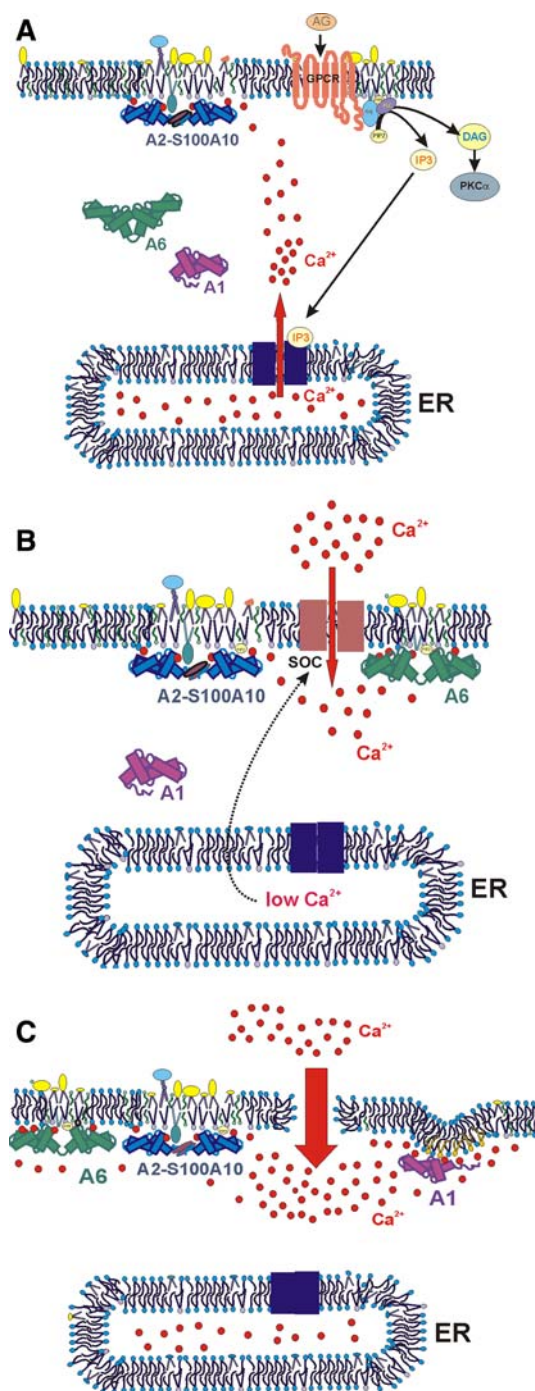

Fig. 5 Annexins discriminate between the magnitude and duration of Ca^{2+} signals. Different Ca^{2+} sensitivities of annexins allow them to interact with the plasma membrane under specific conditions. **a** Ca^{2+} transients caused by IP_3-mediated release from intracellular stores (ER) are sensed by the annexin A2-S100A10 complex, which translocates to the plasma membrane. **b** Store-operated Ca^{2+} entry activated by an empty ER results in the plasma membrane translocation of annexin A6 and annexin A2. **c** Prolonged elevation of $[Ca^{2+}]_i$, occurring during stress and membrane damage, causes PM association of annexin A1, which preferentially binds to ceramide-rich areas

human annexin A5 and hydra annexin B12 were able to bind phospholipid vesicles in vitro at pH 5.3 and 5.8, respectively. Similar interactions were described in further studies of annexins B12 [125, 128] and human annexin A6 [124, 129]. It should be noted that these studies were performed in vitro at pH between 4 and 6, i.e., under conditions that rarely occur in living cells.

We characterized in vivo the influence of intracellular pH on $[Ca^{2+}]_i$ and membrane association of human annexins A1, A2, A4, A5, and A6 tagged with fluorescent proteins [130]. Annexin A6 and the heterotetramer A2-S100A10 (p11)—but not annexins A1, A4, and A5—Ca^{2+}-independently interact with the plasma membrane at pH 6.6 and 6.8. The dimerisation of annexin A2 within the annexin A2-S100A10 complex is essential for the pH-dependent membrane interaction at this pH range. The observations that annexin A6 interacts with membranes of late endosomes [71] and annexin A5 induces pH-dependent vesicle aggregation and fusion [131] point to the fact that the local acidic microenvironment of certain intracellular compartments allows such Ca^{2+}-independent membrane interactions to occur in vivo.

Two types of pH-induced membrane interactions have been proposed: protein insertion into the membrane bilayer, resulting in a transmembrane annexin, and the membrane binding associated with increased hydrophobicity of annexins at acidic pH. Exposure to low pH may cause a significant conformational change in the core domain generating a transmembrane form of the protein as suggested for annexins B12 [125, 132], A5 [127], and A6 [124, 133]. In contrast, no such dramatic rearrangement takes place in the case of annexins A1 and A2 [134]. Under mildly acidic conditions, annexin A2 binds to and aggregates membranes containing anionic phospholipids. Using time-resolved fluorescence analysis and the measurement of Stokes radius, Lambert et al. [134] demonstrated that at mildly acidic pH, the protein undergoes conformational changes similar to those induced by Ca^{2+}. These changes allow access to the hydrophobic part of the membrane at both acidic pH in the absence of Ca^{2+} and at neutral pH in the presence of Ca^{2+}. However, membrane bridging by the annexin A2-S100A10 heterotetramer did not involve further structural modification of the protein core. The NH_2-terminal domain of annexin A2 was implicated in mediating pH-induced membrane aggregation in vitro [135].

The issue of annexins acting as ion channels in their own right is somewhat controversial as such activity was only observed in vitro and usually requires low (below 6.0) pH, which induces extensive alterations of the protein structure. Most annexins might be capable of forming membrane channels in vitro; first described for annexin A7 [136], this feature was observed also for annexins A5 [136], A2 [137], and A1 [138]. The crystallographic studies show a hydrophilic pore at the centre of the annexin molecule, forming a prominent ion channel coated with charged and highly conserved residues [139]. The cation/anion permeability ratios of the ion channels formed by several annexins confirm the selectivity of the annexin channels for Ca^{2+} over other divalent cations and reveal the importance of amino acid positions 17, 78, 95, and 112 for the identification of the ion channel's position, function, and regulation. However, the annexin-mediated ion conductance is reversely proportional to the concentration of Ca^{2+}, and most annexins require high Ca^{2+} for membrane interaction. On the other hand, low pH favors membrane insertion of annexins (see above) and might therefore promote channel activity. Indeed, the mechanism of pore formation at low pH was proposed by Langen et al. [140] based on the membrane-inserted form of annexin B12 at low pH. Upon pH-induced membrane insertion, a continuous transmembrane alpha-helix is reversibly formed from a helix-loop-helix motif seen in the solution structure. The authors identified other regions with similar membrane-insertion potential in the amino acid sequence and proposed that the corresponding helices come together to form an aqueous pore mediating the ion channel activity reported for several annexins [140].

Cell signaling and annexins

Role of annexins in the regulation of intracellular Ca^{2+} homeostasis and modulation of ion channels

Annexin A6 is the most extensively investigated member of the family in terms of its influence on intracellular Ca^{2+} homeostasis and has been implicated in the regulation of the sarcoplasmic reticulum ryanodine-sensitive Ca^{2+} channel [141], the neuronal K^+ and Ca^{2+} channels [142], and the cardiac Na^+/Ca^{2+} exchanger [143]. Studies with transgenic animals delineated an important function of annexin A6 in maintaining intracellular Ca^{2+} homeostasis. Cardiac-specific over-expression of annexin A6 resulted in lower basal $[Ca^{2+}]$, a depression of $[Ca^{2+}]_i$ transients, and impaired cardiomyocyte contractility [144]. In contrast, the cardiomyocytes from annexin A6 null-mutants showed increased contractility and accelerated Ca^{2+} clearance [145]. Consistent with its role in mediating intracellular Ca^{2+} signals, especially Ca^{2+} influx, ectopic overexpression of annexin A6 in A431 cells, which lack endogenous annexin, resulted in inhibition of EGF-dependent Ca^{2+} entry [146].

Store-operated Ca^{2+} entry (SOCE) has been proposed as a main process controlling Ca^{2+} entry in non-excitable cells [147], and the recent discovery of Orai1 and STIM provided a missing link between the Ca^{2+}-release-activated current

(I_{CRAC}) and the ER Ca^{2+} sensor [148–150]. The actin cytoskeleton plays a major role in the regulation of SOCE, possibly by influencing the function of ion channels or by interfering with the interaction between STIM and Orai1 [151–153]. However, the proteins connecting the actin cytoskeleton and SOCE activity have yet to be identified. We targeted annexin A6 to the plasma membrane independent of Ca^{2+} and demonstrated that the constitutive PM localization of annexin A6 resulted in down-regulation of store-operated Ca^{2+} entry (Monastyrskaya et al., unpublished data). Expression of membrane-anchored annexin A6 caused an accumulation of cortical F-actin, and cytoskeletal destabilization with latrunculin A abolished the inhibitory effect of plasma membrane-anchored annexin A6 on SOCE. Our results implicate annexin A6 in the maintenance of intracellular Ca^{2+} homeostasis via actin-dependent regulation of Ca^{2+} entry.

The role of annexins as regulators of ion channel activity has been addressed in a number of studies (reviewed in [154]). In epithelial cells, annexin A4 had an inhibitory effect on Ca^{2+}-dependent Cl^- conductance [155]. Using a specific peptide, corresponding to the NH_2-terminus of annexin A2 and interfering with annexin A2-S100A10 complex formation, it was demonstrated that this annexin heterotetramer could be involved in the function of a volume-activated chloride channel in endothelial cells [156].

Recently, several studies implicated annexins in complex with S100 proteins in the regulation of trafficking and function of several ion channels. Transient receptor potential channels TRPV5 and TRPV6 have an important function in Ca^{2+} uptake by kidney and intestine epithelia [157]. These channels are constitutively active at low intracellular Ca^{2+} and physiological membrane potential and have a high selectivity for Ca^{2+}. They are regulated by associated proteins, including calmodulin, 80K-H, Rab11a, Na^+/H^+ exchanger regulatory factor 2, and the annexin A2-S100A10 complex [158]. S100A10 has been identified as an auxiliary protein for these Ca^{2+} channels using a yeast two-hybrid system [159]. It was shown that annexin A2 heterotetramer was co-expressed with TRPV5 and TRPV6 in Ca^{2+}-transporting epithelia and was essential for their function. Mutation of a sequence mediating the interaction of TRPV5 and TRPV6 with S100A10 caused a major disturbance in the intracellular localization of these channels, indicating that their complex with A2-S100A10 was mediating their correct trafficking. A similar complex was detected in airway epithelia [160], and its formation is regulated by the cAMP/PKA/calcineurin A pathway, which determines the association between annexin A2 and S100A10. The same signaling pathway promotes the interaction of the annexin A2-S100A10 complex with the cystic fibrosis conductance regulator protein (CFTR) and regulates its channel function [161, 162]. An acetylated peptide, corresponding to the NH_2-terminus of annexin A2, harboring the S100A10 interaction site, was shown to interfere with A2-S100A10/CFTR complex formation, inhibiting the CFTR current in epithelia.

Similarly, the TWIK-related acid-sensitive K^+ channel (TASK1) was shown to be associated with S100A10 via its C-terminal amino acids [163]. This sequence (SSV) resembles the binding motif of TRPV channels, suggesting that both proteins share the binding pocket on S100A10. This feature, however, is not essential for the interaction, as the voltage-gated Na^+ channel ($Na_v1.8$) binds S100A10 via an unrelated NH_2-terminus [164]. A dorsal root ganglion-specific knock-out of S100A10 allowed examination of its role in nociception [165]. The expression levels of annexin A2 were not affected, however, the S100A10-null neurons showed deficits in the expression of $Na_v1.8$ and attenuation of acute pain behavior. The annexin A2-S100A10 complex with the acid-sensing ASIC1 channel was detected by immunoprecipitation in dorsal root ganglion neurons [166]. Taken together, these findings indicate that the S100A10-annexin A2 complex plays an important role in the trafficking of several ion channels to the plasma membrane. The regulation of this complex by cAMP/PKA/calcineurin adds another important level of control of channel activity and allows the convergence of different intracellular pathways on the plasma membrane to be influenced by annexins.

Regulation of PKC

Similar to the annexins, PKCα is a phospholipid-binding protein that translocates from the cytoplasm to the plasma membrane upon elevation of $[Ca^{2+}]_i$ [167, 168]. DAG is a major activator of PKC. Addition of DAG to lipid membranes has been shown to increase Ca^{2+}-binding of several annexins, in particular annexin A6 (E. Babiychuk, unpublished data). It is therefore possible that both proteins associate with similar areas of the plasma membrane upon intracellular stimulation and elevation of $[Ca^{2+}]_i$. Indeed, activated PKCα was shown to associate with annexin A6 in skeletal muscle [169]. Unlike annexins A1, A2, and A4, annexin A6 is not phosphorylated by PKCα and might regulate Ca^{2+}-dependent PKC activity, either by targeting the PKC to specific intracellular locations or by competitive inhibition thus enhancing signal termination [169]. Upon activation, PKCα has been shown to translocate preferentially to lipid rafts [170, 171], and a Ca^{2+}-dependent association of PKCα with neurocalcin and annexin A6 has been observed in lipid rafts from the synaptic plasma membrane of rat brain [172]. These findings support the hypothesis that annexin A6 might act as a scaffold for PKC, thus influencing its function.

Annexin A5 has been described as a regulator of PKC activity [173]. In Jurkat cells, annexin A5 inhibited phosphorylation of annexin A2 by endogenous PKC and phosphorylation of myelin basic protein by PKCα [173]. PKC inhibition by annexin A5 was the consequence of phospholipid sequestration, and the Ca^{2+}-binding site located in domain 1 of annexin A5 was shown to play a predominant role in this process. The studies in cell-free systems demonstrated the ability of annexin A5 to inhibit the PKC-catalyzed phosphorylation in the presence or absence of its activator 12-o-tetradecanoylphorbol-13-acetate (TPA) [174, 175]. Overexpression of annexin A5 in breast cancer MCF-7 cells resulted in an inhibition of TPA-induced Ras/ERK signaling via inhibition of PKC, possibly PKCδ, but the exact mechanism of its action has not been elucidated [176].

Recent studies link the function of PKC to the amount of $PtdIns_{4,5}P_2$ in the plasma membrane [177, 178]. An increase in the $PtdIns_{4,5}P_2$ concentration prolongs the presence of PKCα within the plasma membrane when PC12 cells were stimulated with ATP, independently of the DAG generated. When the magnitude of $PtdIns_{4,5}P_2$ binding was compared with that of other polyphosphate phosphatidylinositols, it was seen to be greater in both PKCβ- and PKCγ-C2 domains. The concentration of Ca^{2+} required to bind to membranes was lower in the presence of $PtdIns_{4,5}P_2$ for all C2 domains, especially for PKCα. Considering the preferential binding of annexins A2 and A8 to $PtdIns_{4,5}P_2$ (see above), one might envisage a regulatory cross-roads in the function of these protein families.

Regulation of Ras

The activation of Ras occurs downstream of many cell surface receptors, including receptor protein kinases, and the Ras/Raf/MAPK pathway serves as a link between the signalling cascades regulating cell growth, differentiation, and survival. The Ras GTPases are resident at the inner leaflet of the plasma membrane and function as molecular switches, shuttling between an inactive Ras-GDP and an active Ras-GTP [179]. The GTP/GDP exchange rate is regulated by nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) [180, 181]. Earlier studies established the existence of a specific interaction between annexin A6 and p120GAP [182, 183] and showed that this binding was Ca^{2+}-independent and required amino acids 325–363 of annexin A6, localized in the inter-lobe linker region of the protein. Since this binding site does not interfere with the Ca^{2+}-dependent interaction of annexin A6 with the plasma membrane, it might facilitate the membrane binding of p120GAP, and indeed it was shown that annexin A6 inactivated Ras by recruiting p120GAP to the plasma membrane [184, 185]. In CHO cells, the

elevation of Ca^{2+} following an activation of growth factor receptors promoted a membrane association of the annexin A6-p120GAP complex and consequently a reduction of EGF-induced activation of Ras and Raf-1.

Overexpression of epidermal growth factor receptor (EGFR) is associated with enhanced activation of wild-type (hyperactive) Ras in breast cancer [186]. Recently it was shown that annexin A6 was down-regulated in a number of EGFR-overexpressing and estrogen receptor-negative breast cancer cells [186]. In these cells, the restored expression of annexin A6 promoted Ca^{2+}- and EGF-inducible membrane targeting of p120GAP. The Ca^{2+}- and EGF-inducible complex of annexin A6 with H-Ras was detected by co-immunoprecipitation from the cell lysates of these cells, and FRET microscopy confirmed the close proximity of annexin A6 and the active (G12V), but not inactive (S17N) H-Ras [186]. Interestingly, the expression of annexin A6 is reduced or lost in many cancers [187], which might have a bearing for cancer progression. It is likely that the tumor-suppressing activity of annexin A6 is linked to its ability to cause the down-regulation of the cell proliferation pathways initiated by activated Ras.

Annexins and intracellular stress

Membrane interaction upon hypoxia-induced intracellular acidification

The intracellular pH (pH_i) is an important physiological parameter, and several proteins have the ability to interact with cellular membranes in a pH-dependent manner, thus acting as pH sensors: cytochrome C, Bcl-xL, and chromogranins A and B have been shown to bind lipid vesicles at acidic pH [188–190], and ARF1 is associated with endosomal membranes in a pH-dependent manner [191].

Hypoxia and ischemia are associated with a decrease in pH_i: a mean pH_i of 5.3 was demonstrated in hippocampal neurons [192, 193]. Subjecting the cells to hypoxia induced a robust expression of the oxygen-regulated HIF-1 subunit HIF-1α, observed already after 1 h of exposure, and a concomitant decrease in the pH_i [130]. We showed that hypoxia promoted the interaction of recombinant annexin A2-S100A10 and annexin A6 with the plasma membrane: exposure to hypoxic conditions resulted in an association of annexin A6 with the plasma membrane [130]. Similarly, the annexin A2-S100A10 heterotetramer translocated to the plasma membrane after 30 min of hypoxia. The pH_i values required for membrane association of the recombinant annexin A2-S100A10 in normal and hypoxic conditions correlate well, which indicates that it is the

hypoxia-induced intracellular acidification and not the reduction of O_2 per se that drives the interaction [130].

Intracellular pH values, which in resting cells are in the neutral range (7.0–7.2), can change rapidly during muscle contraction: intramuscular pH declines from 7.1 to 6.5 due to skeletal-muscle fatigue [194]. Hypoxia and ischemia are accompanied by a dramatic drop in pH_i. This decrease probably results from immediate blockade of oxidative phosphorylation and activation of glycolysis [195]. An important consequence of intracellular acidification in neurons is the Ca^{2+}-dependent increase in neurotransmitter release [196]. In the heart, an ischemia-induced decrease in pH_i from 7.4 to 6.7 has recently been shown to activate the acid-sensing ion channels (ASICS) in the sensory neurons and contribute to the sensation of angina [197]. Interestingly, hypoxia induced the expression of annexins A2 and A5 in the placental cell line BeWo, whereas the levels of antioxidants and some cytoskeletal proteins were reduced [198]. Similarly, hypoxia induced the expression of annexin A2 in cultured human cervical epithelial cells [199]. The annexin A2-S100A10 complex, shown to regulate ASICS1 [166] under normal conditions, might therefore have an additional function during hypoxic stress, thus indirectly contributing to the regulation of pH-induced ion channel activity. Alternatively, annexin A6, recruited to the plasma membrane by a decrease in pH_i, might influence the pH homeostasis through its interaction with PKC, a known regulator of the activity of the NHE1 proton exchanger.

Cellular stress and membrane repair

Hypoxia, osmotic and heat shock, oxidative stress, and local membrane damage result in the activation of multiple intracellular signaling pathways, accompanied by changes in $[Ca^{2+}]_i$ and leading ultimately to adaptive changes in gene expression. Oxidative stress has been shown to affect expression and localization of different annexins. In earlier publications, annexin A2 was identified as an oxidant-sensitive protein, based on a screen of transient incorporation of glutathione into cellular proteins [200]. Glutathionylation inhibited the membrane-binding activity of annexin A2 and might represent a regulatory mechanism influencing its function at the plasma membrane [201]. In contrast, annexin A5 was shown to associate Ca^{2+}-independently with phospholipid vesicles in vitro at pH 7.4, in the presence of 3 mM H_2O_2 [202]. The expression and localization of annexins was investigated in chronically hyperoxically stressed endothelial cells [203]. In this study, annexin expression levels did not change significantly in response to hyperoxic stress, but immunofluorescence analysis revealed striking effects on the subcellular localization of certain annexins, including the redistribution of annexins A5 and A6 from the cytosol to the nucleus.

Annexins have been implicated in the regulation of repair mechanisms on both tissue and intracellular levels. Annexin A2 was implicated in wound closure in several studies. Patchell et al. [204] examined the role of glycosylation and expression of glycoproteins after epithelial injury and demonstrated that following injury, N-glycosylation events and annexin A2 presentation on the cell surface of airway epithelial cells are important mediators in repair [204]. Following mechanical wounding of confluent monolayers of human airway epithelial cells, glycosylation staining increased on the cell surface of groups of cells in the proximity of the wound edge. By membrane protein biotinylation and immunodetection, the authors have shown that following mechanical wounding, the presentation of annexin A2 on the cell surface increased coordinately with repair. Babbin et al. [205] observed that gastrointestinal mucosal wound healing is dependent on actin cytoskeletal reorganization and that there is an increased expression of annexin A2 in migrating intestinal epithelial cells. The authors used siRNA-mediated inhibition of annexin A2 expression and observed Rho dissociation from membranes and decreased Rho activity following annexin A2 siRNA transfection. Annexin A2 was observed to co-immunoprecipitate with endogenous Rho and constitutively active RhoA. These findings suggest that annexin A2 plays a role in targeting Rho to cellular membranes during epithelial cell migration and tissue repair [205].

Annexin A1 also has an important function in intracellular repair and tissue regeneration mechanisms. Using annexin A1-knockout mice, Babbin et al. [206] demonstrated the role of this annexin in the regulation of intestinal mucosal injury and repair: annexin A1-deficient animals exhibited an increased susceptibility to dextran sulfate sodium-induced colitis with greater clinical morbidity and histopathologically visible mucosal injury [206]. At the intracellular level, both annexins A1 and A2 were shown to interact with dysferlin in a Ca^{2+}- and membrane-injury-dependent manner [207]. The distribution of the annexins and the efficiency of sarcolemmal wound-healing are significantly disrupted in dysferlin-deficient muscle, suggesting an important role of annexins in sarcolemmal repair through mediating membrane fusion.

McNeil et al. [208] established a role of annexin A1 in membrane repair. They showed that Ca^{2+} entering a cell through a damaged plasma membrane rapidly triggers membrane fusion events, resulting in the assembly of a reparative membrane patch at the defect site. An annexin A1 function-blocking antibody, a small peptide competitor, and a dominant-negative annexin A1 mutant protein incapable of Ca^{2+} binding, all inhibited resealing. Coincident with a resealing event, annexin A1 was observed to concentrate at disruption sites. The authors propose that Ca^{2+}

entry through a disruption locally induces annexin A1 binding to membranes, initiating emergency fusion events whenever and wherever required.

Annexins as diagnostic tools for human disease

Although there is no direct evidence implicating any individual member of the annexin family as a disease-causing gene, in certain clinical conditions, changes in annexin expression levels or localization may contribute to the progression of disease. In this way, annexins are indirectly linked to some of the most serious human pathologies including cardiovascular disease and cancer [41, 209]. Despite a wealth of experimental data, in the case of these "annexinopathies," the translation of scientific results into therapeutic concepts is still in its infancy.

In the meantime, the importance of annexins as diagnostic tools is becoming increasingly recognized. Coupled to thrombolytic enzymes, annexin A5 has localized intravascular thrombi [210]. Technetium-labeled annexin A5 has been used as a marker for apoptotic cells in cardiovascular medicine [211], and its specific binding to phosphatidylserine has made this protein a potential candidate for predicting the effectiveness of cancer chemotherapy [212]. Annexin A1, which has been credited with anti-inflammatory properties [213], is thought to contribute to the resolution of an inflammatory response by the removal of extravasated neutrophils [214]. Therefore the ability of annexins to respond to disease- and stress-related alterations of the cellular environment and restore the intracellular homeostasis makes this protein family an important group of diagnostic and therapeutic targets.

Conclusions

A chronic stress response triggers the activation of different intracellular pathways, resulting in profound changes in Ca^{2+} and pH homeostasis, production of lipid second messengers such as $PtdIns_{4,5}P_2$, DAG, and ceramide, and an alteration of membrane curvature caused by clustering of specific lipids. These changes are sensed by the members of the annexin protein family, which have the ability to interact with specific lipid moieties at the plasma membrane and simultaneously associate with various signaling proteins.

Indeed, annexins act as sensors for $[Ca^{2+}]_i$. They interact with the plasma membrane and other cellular membranes in a coordinated, Ca^{2+}-dependent manner. The Ca^{2+} sensitivity of annexin-membrane binding is affected and modulated by interaction with other proteins and proteolytic cleavage, and several annexins present within any one cell form a broad-range $[Ca^{2+}]_i$-sensing system.

Additionally, annexins respond to pH_i decrease, and specifically annexins A6 and A2-S100A10 act as pH sensors by interacting with the plasma membrane of living cells upon a decrease in pH_i to 6.6 and 6.8. The pH-induced membrane interaction of annexins is Ca^{2+}-independent and, together with the ability of annexins to sense changes in membrane curvature, represents an important alternative mechanism of membrane binding.

Intracellular acidification, oxidative stress, and a concomitant increase in $[Ca^{2+}]_i$ promote membrane binding of annexins, which have distinct preferences as to the proteins and lipids with which they interact. Annexins A1, A2, and A6 act as sensors for lipid second messengers ceramide, $PtdIns_{4,5}P_2$ (PIP_2), and DAG. Upon membrane binding, annexins regulate various signaling pathways, including the activation of PKC and Ras, and regulate Ca^{2+} homeostasis by affecting ion channels and Ca^{2+} entry. Importantly, they also affect the mechanism of intracellular membrane repair in a Ca^{2+}-dependent manner, thus defining cell survival and proliferation under stress conditions.

The high degree of sequence conservation, and considerable functional and structural redundancy of different annexins renders them important regulators of cell signaling. Novel functions of individual members of the annexin protein family, which have recently been elucidated, show the diversity of these proteins. On the other hand, these specific functions might be executed in parallel with a more general role of annexins as watchdogs of intracellular homeostasis, contributing to cellular adaptation to stress signals.

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