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Annexins in the secretory pathway

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Abstract. Among the multiplicity of roles suggested for proteins of the annexin family, those implicating these proteins in regulated exocytosis remain among the most convincing. Studies in this area of annexin biology have focused on annexin II, which because of its unusually low Ca^{2+}-requirement for phospholipid-binding has many of the requisite properties of a membrane fusogenic Ca^{2+} sensor. Other annexins are also good candidates for exocytotic mediators, especially annexins I and VII, which have strong vesicle-aggregating activities. In contrast, annexin VI appears to block vesicle aggregation, perhaps acting as a negative regulator of exocytosis. In this review, we consider the evidence for and against annexins having functions in the secretory pathway.
Key words. Annexin; secretion; vesicle; exocytosis; calcium; neutrophil; chromaffin cell.

Introduction

Intercellular communication is one of the basic tenets of cell biology. For multicellular organisms this is especially important, in order to coordinate the activities of the various cell types. Central to this exchange is the process of exocytosis, whereby intracellular components such as proteins or peptides are sequestered in membrane vesicles, shuttled to the plasma membrane and liberated from the cell by vesicle fusion. Eukaryotic cells carry out two forms of exocytosis: constitutive and regulated. In the former, vesicle formation is followed by immediate fusion, while in the latter, vesicles may wait in the cytoplasm for a signal before fusion is initiated. Whereas constitutive exocytosis may be common to virtually all cell types, regulated exocytosis is thought to be restricted to a rather narrow range such as endocrine and neuronal cells [1].

Exocytosis may be triggered by the activation of membrane-bound proteins such as the FcER1 receptor on the surface of mast cells, or by depolarization of the plasma membrane, which initiates the release of neurotransmitter from the presynaptic terminal. In both cases, vesicle fusion follows a rise in the concentration of free cytosolic calcium, either from internal stores such as the endoplasmic reticulum, or from the extracellular space, via integral membrane calcium channels. In the latter case, the rise in calcium may be restricted to localized regions immediately beneath the plasma membrane. This could have important implications, preventing vesicles in the cell interior from docking and fusing with the membrane. In permeabilized adrenal chromaffin cells, maximal stimulation of secretion occurs at around 10 μM free calcium [2]. A similar calcium requirement has since been shown for a variety of other cell types and work is under way in laboratories worldwide, to identify the proteins which respond to this calcium rise and mediate vesicle docking and fusion.

The finding that adrenal medullary chromaffin granules aggregate and fuse in the presence of cytosolic extract led to the discovery in 1978 of the 55 kD protein synexin which promotes vesicle aggregation in vitro, in the presence of 200 μM free calcium [3]. Some ten years later, molecular cloning [5] revealed synexin to be a member of a large family of proteins now termed the annexins. Like all members of the family, synexin (annexin VII) binds calcium-dependently to acidic, negatively charged phospholipids, such as those enriched in the inner leaflet of the plasma membrane and the cytoplasmic aspect of cellular organelles such as secretory vesicles. Annexins differ from the other major family of calcium-binding proteins exemplified by calmodulin, that bind calcium via the 'E-F hand' helix-loop-helix structure. Annexins bind calcium via a conserved 70 amino acid sequence termed the 'annexin core' [5]. This motif forms the structural feature that defines annexins. Each annexin contains the core sequence repeated four times at the C-terminal half of the protein, the only exception to this rule being annexin VI, in which the motif is repeated eight times. It is highly likely that the repetitive annexin core arose by gene duplication, since there is strong homology between the repeats in any one annexin and also between the different members of the family. The best characterized role of the annexin core underlies that property which is common to all annexins, calcium and phospholipid binding. This notion is supported by crystallographic studies and by experiments in which mutation of residues within these domains was shown to compromise calcium-dependent binding to phospholipids.

At the N-terminus of the proteins, there is no significant sequence similarity between family members. Indeed the

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so-called 'tail domain' varies from 10–18 residues up to more than 100. In light of this sequence divergence, and also since the tail domain contains residues for tyrosine and serine phosphorylation, as well as other post-translational modifications, it has been postulated that this region confers the properties of the protein which are specific to each family member [6]. The N-termini of a number of annexins contain binding sites for cellular protein ligands, which may be important for function. For example, annexin I and II N-termini contain binding sites for the E-F hand containing S100 proteins (S100C and p11 respectively). In the case of annexin I, it is likely that S100C binding requires elevation of intracellular calcium. However, it has been shown that mutation of key residues within the E-F hands on p11 have rendered the protein incapable of binding calcium, which means that the annexin II/p11 heterotetrameric complex is stable even in the absence of calcium.

Proposed functions for annexins

In considering whether or not annexins have roles to play in the secretory pathway, it is important to view such roles in the context of other abundant functional theories. These include control of intracellular calcium mobilization, regulation of vesicle trafficking, inhibition of phospholipase A_2, and inhibition of blood coagulation. Among these diverse activities, the evidence for an involvement of at least one of the family members in the exocytotic pathway is especially compelling [7].

As already stated, annexin VII (synexin) can promote chromaffin cell vesicle fusion in vitro, albeit at unphysiologically high levels of free calcium. However, the protein seems to be involved mainly in docking the vesicle to the membrane, since vesicle fusion occurs rather slowly in vitro. But in the presence of small amounts of cis-unsaturated fatty acids (such as arachidonic acid), the rate of annexin-mediated membrane fusion is increased dramatically. This is intriguing since one of the most contentious aspects of annexin biology is the proposal that annexin I inhibits cytosolic phospholipase A_2 [8], which is known to release free arachidonic acid from the cell membrane.

Since the early work of Creutz et al. [3] a number of studies have been performed which strongly suggest that annexins play a significant, perhaps critical role in secretion. In this review, we discuss the roles of annexins as membrane fusogens, Ca^{2+} sensors, and signal transducers in regulated exocytosis.

Annexin I

Annexin I was the first member of the annexin family to be cloned. Although the majority of work on this protein has focused on its proposed function as a phospholipase A_2 inhibitor, a number of studies implicate annexin I in secretion.

Annexin I and insulin secretion. Insulin secretion from the β-cells in the islets of the pancreas is a tightly regulated process. One of the effects of the secretagogue, glucose, is to elevate cytosolic calcium levels prior to insulin secretion. The proteins that are involved in detecting and modulating the response to this transient calcium rise are not well characterized. However, the work of Ohnishi et al. strongly suggests that annexin I plays a pivotal role in this process [9]. Using antibodies to annexin I and insulin tagged with different sized gold particles, immunoelectron microscopy localized these proteins to the rat pancreatic islets. Annexin I was shown to be strongly colocalized with insulin-containing granules. Furthermore, when glucose-treated rats were compared with those starved overnight, there was a significant increase in the level of annexin I associated with insulin granules, suggesting that, when β-cells are stimulated to secrete insulin, the process requires the translocation of annexin I to the insulin-containing vesicles.

An interesting addendum to this study is that glucose treatment of isolated islets was shown to induce a dramatic increase in the level of annexin I phosphorylation, concomitant with the burst of insulin secretion. Analysis of annexin I phosphorylation sites revealed that the glucose-stimulated phosphorylation of the protein occurred predominantly on serine residues. Annexin I is known to contain potential sites for phosphorylation by protein kinase C (PKC) [10]. To test the involvement of this enzyme, the potent pharmacological inhibitor of PKC, H7, was applied to isolated islets prior to glucose treatment. Not only was annexin I phosphorylation reduced by up to 90%, but there was also a significant reduction in the level of insulin secretion. However, it must be noted that the depression in insulin secretion was not nearly as great as the level of PKC inhibition, suggesting either that only a low level of annexin I phosphorylation is required to stimulate some degree of insulin secretion, or that other factors as well as annexin I are involved. Finally, a further observation implicating annexin I in insulin secretion was the discovery that in diabetic rats, the levels of annexin I in pancreatic islets was severely decreased. Their lack of glucose responsiveness could be a functional consequence of this reduction.

Annexin I and neutrophil degranulation. Neutrophil degranulation is essentially a secretory response, and is preceded by a transiently elevated cytosolic calcium level following stimulation of neutrophils with various agonists. It is quite likely that in neutrophils, as in other secretory cells, the calcium rise actually mediates the membrane-fusion events that are an integral part of the degranulation process. The identity of the proteins involved in the calcium-triggered membrane fusion has yet to be determined. However, in an elegant series of experiments, Meers et al. propose a critical role for annexin I in this process [11].

These authors used spectrofluorometric techniques to measure the calcium-dependent aggregation and fusion of fluorescently labelled synthetic phosphatidylethanolamine/phosphatidylserine liposomes with human neutrophil membranes. Although fusion was minimal in the presence of 1 μM free calcium, addition of increasing amounts of neutrophil cytosolic extract markedly enhanced vesicle/membrane fusion, consistent with expectations. Parallel experiments replacing the cytosolic extract with either recombinant annexin I or annexin I purified from neutrophils resulted in significantly greater levels of fusion than that achieved with cytosol alone. This strongly suggests that annexin I is a major secretory modulator in neutrophils, and that in cytosolic extracts, inhibitory proteins may downregulate annexin I-mediated secretion. Interestingly, a recombinant form of annexin I in which the N-terminal 9 residues had been truncated showed essentially no ability to stimulate vesicle aggregation. It is possible that this deletion impairs structural features of the protein necessary for vesicle aggregation, perhaps by eliminating the S100C binding site. Addition of a range of monoclonal antibodies specific for either the annexin I N-terminus or the first repeat completely abolished vesicle aggregation due to the addition of cytosol or purified annexin I, but had no effect on the low level of intrinsic vesicle fusion observed in the absence of added factors.

As with annexin VII, the exact nature of the role of annexin I in this system is open to question. Does the protein induce vesicle aggregation alone or does it also trigger membrane fusion? In the presence of calcium alone, the level of aggregation is the rate-limiting step and this is significantly enhanced by the addition of annexin I. However, in the presence of both calcium and magnesium, vesicle fusion is the rate-limiting step. In this system, the addition of annexin I does not increase the rate of vesicle fusion, and even appears to be inhibitory at high concentrations. The mode of annexin I binding to the vesicles was analysed by following changes in the fluorescence of labelled phospholipids on the addition of annexin I. Preincubation of annexin I with a monoclonal antibody directed to the N-terminal region did not prevent the protein binding to the vesicles, but did abolish calcium-triggered vesicle aggregation, suggesting that the N-terminal domain is directed away from the membrane as the protein binds. By crosslinking bound annexin I to aggregating vesicle lipids using photoaffinity labels, it was shown that a single annexin I molecule is able to bind two lipids on two membranes simultaneously. This suggests that vesicle aggregation may not be mediated by protein-protein interactions as previously assumed, but rather by ligation of two closely apposed phospholipid membranes by a single protein unit.

Annexin I and the pigeon cropsac. Further evidence implicating annexin I in the secretory process emerged from studies on prolactin-inducible genes in the pigeon cropsac [12]. Prolactin induces the secretory epithelium of the cropsac to undergo differentiation and proliferation, secreting a milk-like substance which nourishes newly-hatched young. The genome of Columbidae contains two separate genes (*cp35* and *cp37*) encoding proteins with strong homology to mammalian annexin I. Although structurally very similar, cp35 and cp37 differ in their N-termini: whereas in cp37 the sites for phosphorylation by the epidermal growth factor receptor tyrosine kinase and protein kinase C are intact, in cp35 these sites are absent, reflecting probable differences in their mechanism of regulation. In the cropsac, cp37 is constitutively expressed whereas cp35 is absent. However, in the presence of prolactin, cp35 is rapidly and strongly induced.

This suggests that cp35 plays a major role in the secretory function of the cropsac. An intriguing possibility is that the induced non-phosphorylatable cp35 functions as a dominant negative inhibitor of its constitutively expressed cellular partner, cp37. If cp37 has a role similar to the endocytic function suggested for mammalian annexin I, then it might be involved in the internalization and intracellular trafficking of activated growth factor receptors. Inhibition of this activity by cp35 could change cellular responses (such as secretion) to growth factors by altering the kinetics of receptor downregulation. In contrast to the studies described above, suggesting direct involvement of annexin I in neutrophil degranulation, this theory would give annexin I an indirect role in the secretory process in the cropsac.

Annexin II

Most studies on the role of annexin II in the secretory process have focused on the adrenal medullary chromaffin cell. Following stimulation with physiological agonists, these cells secrete catecholamines, a process requiring the opening of membrane calcium channels and an elevation in cytosolic calcium from around 0.1 μM to 0.5–10 μM. Only proteins that are activated at this level of calcium can realistically be proposed to function as calcium-triggered mediators of secretion [13]. This proviso would preclude, for example, annexin VII (synexin), which was shown by Creutz et al. to require up to 200 μM free calcium to mediate vesicle aggregation [7]. All annexins are responsive to calcium, but annexin II has been shown to have the lowest calcium requirement for binding to phospholipid vesicles. This makes annexin II perhaps the most attractive candidate for mediating calcium-triggered exocytosis. A range of studies has provided compelling evidence that this is indeed the case.

Run-down assays. The study of regulated secretion has been greatly advanced by the development of the so-called 'run-down' assay. The addition to secretory cells of a permeabilizing agent, commonly streptolysin-o or digitonin, results in soluble cytosolic constituents being gradually released from the cell. This will include factors essential for exocytosis such as proteins, and also small molecules, such as ATP and GTP, the latter being essential for G-protein-mediated secretion. The secretory capability of the cells can be partially or totally restored by adding back selected cytosolic components. By serial refinements of the cytosolic extract, secretory mediators may be identified. Unfortunately, one of the drawbacks of this strategy is that since secretion is a complex, multifactorial system, secretory proteins may be active only in the presence of other unidentified factors and therefore may appear inactive when added back in the absence of other proteins. However, a number of proteins that play a critical role in regulated exocytosis have been identified using this technique.

In the presence of 0.3 μM free calcium, annexin II had no discernible effect on the secretory capability of permeabilized chromaffin cells [14]. This is consistent with the known properties of annexin II, since the protein would not be expected to be membrane-bound at such a low level of calcium. However, when the calcium level is raised to 10 μM, annexin II can almost fully restore the secretion level of permeabilized cells. At this level of calcium, annexin II would be expected to be bound to membranes in the cell, and therefore in the correct cellular compartment to trigger exocytosis. It was further found that both monomeric and tetrameric (i.e. annexin II_2/p_{112}) forms of the protein were similarly active in this reconstitution assay.

However, this supposed activity of annexin II appeared to be contradicted by the study of Wu and Wagner [15]. Again, using bovine adrenal medullary cells in the digitonin permeabilisation run-down assay, they attempted to repeat the findings of earlier studies. In the first part of their study, they showed that the addition of cytosolic constituents to run-down cells restored their secretory capability, a result consistent with previous work. They next attempted to deplete the cytosolic extract of annexin II by passing it through a resin of immobilized anti-annexin II antibody. The annexin II-depleted extract appeared to be as active as the intact cytosol in restoring secretion, suggesting that annexin II plays at best a modulatory role in exocytosis. However, the efficacy of depletion of annexin II from the cytosolic extract is open to question. No depletion process can ever be 100% effective, and it is quite possible that rather small amounts of annexin II protein are capable of restoring secretion to permeabilized cells.

Phosphorylation of annexin II. Reversible protein phosphorylation is central to the control of a multitude of essential cellular activities, including exocytosis. When cells are stimulated to undergo secretion, there appears to be a significant increase in the level of phosphorylation of some proteins, including a number thought to be associated with chromaffin granules [16]. However, dephosphorylation is also of considerable importance; IgE-mediated secretion from mast cells was found to be strongly inhibited by the phosphatase inhibitor okadaic acid [17]. Since annexin II has been shown to have a number of residues that are targets for cellular protein kinases, it is quite likely that any putative secretory function of the protein will be strongly influenced by phosphorylation.

Interest in annexin II was initially generated by the discovery that the protein is one of the main cellular targets for phosphorylation on tyrosine by the $pp60^{v\text{-}src}$ transforming tyrosine kinase [18]. It has since been shown that tyrosine-23 in the N-terminal is susceptible to phosphorylation by the oncogenic products of several transforming viruses [19]. The significance of tyrosine-phosphorylation to the proposed role of annexin II as a secretory mediator is not clear. It has been shown that following phosphorylation by $pp60^{v\text{-}src}$, the binding of annexin II to phospholipid vesicles is reduced [20]. This effect is apparent at all concentrations of calcium and does not seem to influence the association between annexin II and its cellular ligand p11. However, analysis of annexin II from *Xenopus* reveals that although this protein shares 80% homology with its mammalian counterpart, the residue at position 23 is a leucine rather than tyrosine [21]. This suggests that, in *Xenopus* at least, regulation of annexin II function is independent of any requirement for tyrosine phosphorylation at this site.

As well as being a substrate for phosphorylation by tyrosine kinases, annexin II has been shown to be phosphorylated on various serine residues by cellular serine/threonine kinases. One of these residues, serine-25, is a target for phosphorylation by protein kinase C. Phosphorylation at this site does not affect the ability of annexin II to bind to phospholipid vesicles, but does decrease the level of annexin II-mediated vesicle aggregation [22]. The importance of PKC-mediated serine phosphorylation was also investigated by Sarafian and co-workers [23]. They showed that in permeabilized cells treated with staurosporine (a PKC inhibitor), addition of annexin II failed to restore secretory activity. However, when pre-phosphorylated in vitro by purified PKC, annexin II was able to sustain secretory activity.

Annexin II also has a serine residue within the p11-binding site flanked by residues which closely resemble a kinase consensus sequence. This has been proposed to be the site for phosphorylation by CaM- and cAMP-dependent kinases as well as by PKC. This could have serious consequences for the ability of p11 to interact with its binding site on annexin II, which in turn may compromise or even abolish the ability of annexin II to mediate the aggregation of secretory vesicles.

Annexin II and the cytoskeleton. There is growing evidence that the cytoskeleton plays an important and dynamic role in exocytosis, rather than having a passive, simply structural role. Central to the organization of the cytoskeleton is actin, which forms a layer or 'cell-web' beneath the plasma membrane. Electron micrographic studies using rat anterior pituitary secretory cells have shown that secretory granules are located beneath the subcortical actin layer and appear to undergo fusion with the plasma membrane only where the actin layer is absent, suggesting that actin disassembly may be an essential prerequisite for exocytosis [24]. Interactions have been reported between annexin II and components of the cytoskeleton, in particular actin. Ikebuchi et al. reported that the annexin $II_2/p11_2$ tetramer may catalyse calcium-dependent bundling of F-actin, at micromolar calcium levels [25]. Further, sequence analysis of annexin II revealed that the region between residues 286 and 294 is strongly homologous to the actin-binding domain of myosin and is therefore an attractive candidate for mediating actin-bundling. Also, when actin is pre-incubated with a peptide corresponding to annexin II residues 286–294, the actin-bundling activity of the annexin $II_2/p11_2$ tetramer is abolished [26]. These studies provide interesting clues suggesting that, by interacting with cytoskeletal proteins, annexin II may clear a path for secretory vesicles to move to the membrane, allowing fusion to occur.

Clearly, to gain further insight into the role of annexin II in exocytosis, it is important that experimental design progresses to the use of whole cell, rather than permeabilized cell, models. In preliminary experiments in rat basophils, we have succeeded in selectively downregulating annexin II using stable expression of antisense (Upton and Moss, unpublished observations). Unfortunately, the resulting phenotype appears to be seriously disadvantageous to the cells, so that they upregulate levels of endogenous annexin II mRNA to compensate. The development of inducible vectors, particularly the tetracycline-based system, could circumvent this. If annexin II levels could be transiently and reversibly downregulated, the subsequent effects on exocytosis could be analysed without the drawbacks inherent in the run-down assay.

Annexin VI

Annexin VI is unique among the annexin protein family. While other annexins contain four copies of the 'annexin repeat', annexin VI has eight, possibly as the result of gene duplication [27]. The unique structure of annexin VI almost certainly determines the function of the protein and, as discussed below, may explain the activity of annexin VI in secretion. Annexin VI has been implicated in a number of cellular functions, including cell growth regulation and intracellular calcium homeostasis. Its role in secretion has not been extensively investigated, but the available evidence suggests that annexin VI *inhibits* rather than potentiates the secretory process. While exploring the ability of purified annexins to fuse chromaffin vesicles, Zaks and Creutz showed that annexin VI inhibits vesicle aggregation and fusion mediated by annexins II and VII [28]. The exact mechanism of this inhibition is unclear. If vesicle aggregation depends upon the formation of contacts between annexin molecules on neighbouring membranes, it is conceivable that annexin VI may disrupt this interaction, perhaps by competing with annexin II for critical binding sites. Further studies are required to clarify this inhibitory effect.

The pattern of tissue distribution of annexin VI also suggests that it could have a negative regulatory role in exocytosis. It has been shown using a variety of techniques, including immunocytochemistry and immunogold electronmicroscopy, that the annexin VI protein is distributed widely within a variety of organs and cell types, including those involved in the secretion of hormones [29]. Interestingly, the protein was found in a number of secretory epithelia including the sweat glands and the ductal epithelial cells of the salivary glands. However, more striking evidence that annexin VI may inhibit secretion was revealed by its pattern of expression in mammary gland ductal epithelial cells. Normal, non-lactating breast epithelial cells stain strongly for annexin VI, but in similar sections taken from lactating breast, annexin VI is undetectable. This suggests that prolactin acts to downregulate annexin VI, possibly at the level of the promoter. This would be consistent with a role for the protein in inhibiting secretion, since its downregulation would facilitate secretion of breastmilk. On the other hand, annexin VI is upregulated in T and B lymphocyte ontogeny. Immature cells of both lineages, in the thymus and lymph node germinal centres respectively, do not express detectable annexin VI, whereas circulating T and B lymphocytes express high levels of annexin VI. The appearance of annexin VI in these cells correlates, in both cases, with the acquisition of a secretory phenotype. Clearly, secretion from T and B lymphocytes is an extremely finely controlled process, and to have proteins that act as negative regulators of secretion provides the cell with a more sophisticated system of control than would exist with positive secretory mediators alone. It is conceptually sound that if annexin VI functions as a secretory inhibitor, it would be 'switched off' in lactating breast where a strong secretory stimulus is desirable, and 'switched on' in cells of the immune system where a fine level of secretory control is necessary. It will be interesting to determine whether annexin VI levels are downregulated in sweat gland epithelia in response to hormonal signals.

What is the molecular basis of inhibition of secretion by annexin VI? A clue may lie in the structure of the

protein. If annexins II and VII are essential for secretion, it is not inconceivable that annexin VI may act as a competitive inhibitor by occupying interaction sites on vesicles in the secretory pathway. The crystal structure of annexin VI places the two four-repeat lobes approximately perpendicular to one another [30]. So whilst the annexin II heterotetramer may form a bridge between the faces of two adjacent vesicles, annexin VI, with its unique spatial configuration, could disrupt the construction of this bridge by competing for annexin II binding sites on the lipid wall.

Conclusions

It is now nearly twenty years since the initial discovery of synexin (annexin VII) by Creutz et al., in 1978 [3]. Since that time, we have come close to understanding the roles these proteins play in vivo. The prospect of annexin knock-out mice should provide experimental systems in which the full gamut of functional theories for annexins can be tested. The data from such models will prove invaluable in revealing the role of annexins in a range of cellular functions, particularly exocytosis. It is no exaggeration to say that the field may be approaching something of a 'golden age' in which the murky waters of annexin research should clear.

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