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Annexin II tetramer: structure and function

David M. Waisman

Department of Medical Biochemistry, Faculty of Medicine, University of Calgary, Calgary, Alberta, T2N4Nl, Canada

Abstract

The annexins are a family of proteins that bind acidic phospholipids in the presence of Ca^{2+}. The interaction of these proteins with biological membranes has led to the suggestion that these proteins may play a role in membrane trafficking events such as exocytosis, endocytosis and cell-cell adhesion. One member of the annexin family, annexin II, has been shown to exist as a monomer, heterodimer or heterotetramer. The ability of annexin II tetramer to bridge secretory granules to plasma membrane has suggested that this protein may play a role in Ca^{2+}-dependent exocytosis. Annexin II tetramer has also been demonstrated on the extracellular face of some metastatic cells where it mediates the binding of certain metastatic cells to normal cells. Annexin II tetramer is a major cellular substrate of protein kinase C and $pp60^{src}$. Phosphorylation of annexin II tetramer is a negative modulator of protein function. (Mol Cell Biochem **149/150**: 301–322)

Key words: annexins, phosphorylation, calcium binding, phospholipids, membrane bridging, cell-cell interaction, DNA polymerase

Perspectives and overview

The annexins (reviewed in [1–11]) are a family of Ca^{2+}-binding proteins which bind to acidic phospholipids, and are further identifiable as members of this family, by the presence in each of the proteins, of a region of amino acid homology called the 'annexin fold' [12]. The name annexin [13] which originates from the property of the members of this family to annex phospholipid, has been suggested as a basis for a common nomenclature for these proteins [14]. To date, thirteen annexins have been purified and characterized and these proteins have been described in all mammalian cells except erythrocytes. Annexins have also been described in organisms ranging from mammals to molds and plants. Most annexins are abundant intracellular proteins and may comprise about 0.5–2% of total cellular proteins. Typically, the annexins are 30–40 kDa monomeric proteins with the exception of monomeric annexin VI (68 kDa) and annexin II which can exist in monomeric or oligomeric forms. The ability of the annexins to bind to biological membranes in a Ca^{2+}-dependent manner [15] has led to speculation that these proteins might be involved in a number of membrane trafficking events, such as endocytosis [16] or exocytosis [17–24]. A variety of other functions have been described for the annexins and these include transmembrane ion channels [25–29], inhibitors of phospholipase A_2 [7, 30–37], inhibitors of blood coagulation [38–50], transducers of signals for differentiation or mitogenesis [51–62], regulators of cell-matrix interactions [63–69] and regulators of cell-cell adhesion [70–72].

Annexin II (AII) is an abundant annexin which has been shown to exist as a monomer (AIIm, 36 kDa), a heterodimer (AIId) or a heterotetramer (AIIt). The heterodimer is composed of one subunit of AII and one subunit of 3-phosphoglycerate kinase [73] and the heterotetramer is composed of two AII subunits and two 11 kDa subunits [74–76]. The AII monomer is mainly cytosolic. The formation of the heterodimer results in the association of the complex with the nucleus where it has been shown to regulate DNA polymerase α [51, 73, 77, 78]. In contrast, the formation of the heterotetramer results in the association of the complex with the plasma membrane [79]. The AII gene is growth-regulated [53, 80] and the expression of AII is a primary response to mitogenic stimulation. Although the physiological function of AIIt has not been unequivocally established, a role for the protein in Ca^{2+}-dependent exocytosis [17–22, 24, 81, 82], endocytosis [16, 83] and cell-cell adhesion [71, 72, 84] has been suggested.

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Address for offprints: D.M. Waisman, Department of Medical Biochemistry, Faculty of Medicine, University of Calgary, Calgary, Alberta, T2N4Nl, Canada

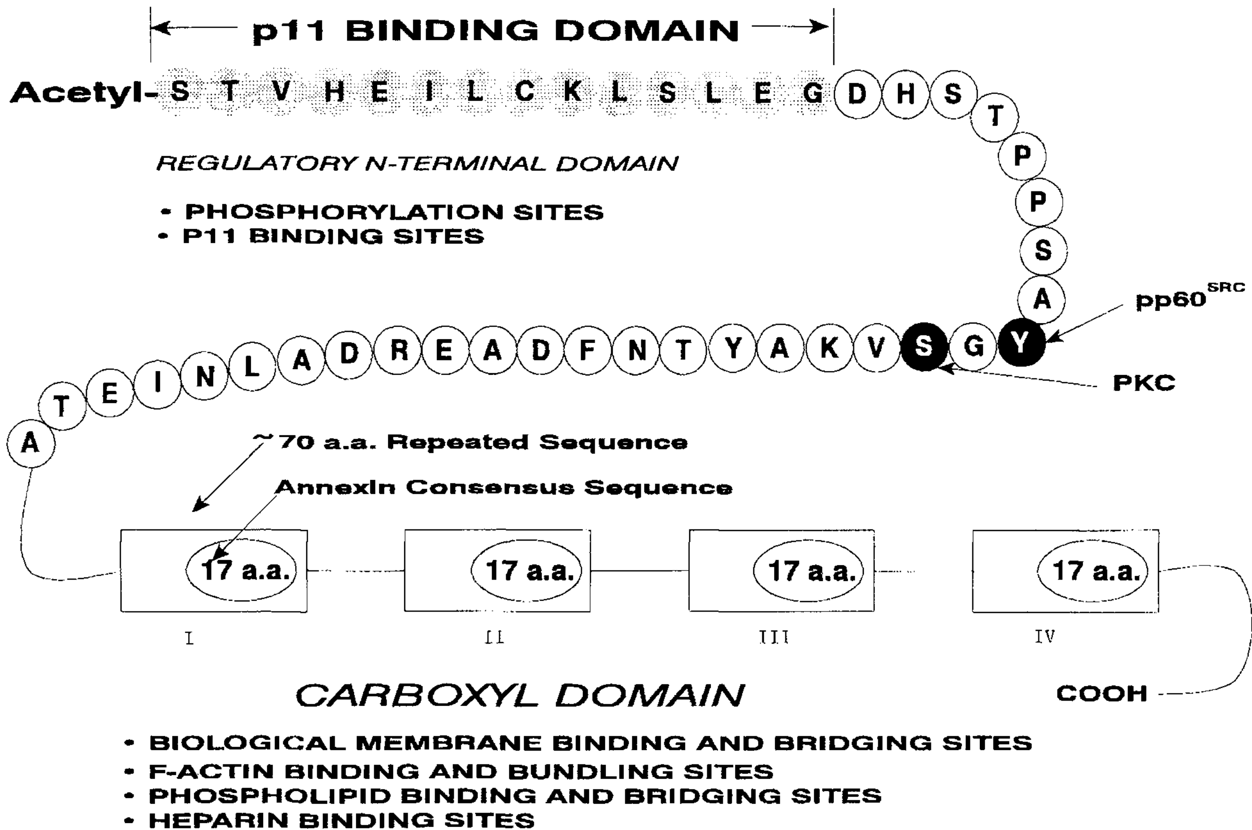


Fig. 1. Annexin II domain structure. A diagrammatic illustration of the domain structure of annexin II is presented. The first 30 residues of the protein comprise the amino-terminal domain while the remainder of the molecule comprises the carboxyl-terminal domain. The amino-terminal contains the p 11 subunit binding sites, the phosphorylation sites for $pp60^{src}$ and protein kinase C (PKC). The carboxyl-domain of the protein contains the phospholipid, F-actin, and heparin binding sites of the protein. The carboxyl-domain is organized in the 4 repeating domains of amino acid sequence homology each of which contains the annexin consensus sequence. The amino terminal domain regulates the heparin binding and F-actin binding properties of the protein as well as the membrane bridging activity of the protein. The amino terminal domain does not appear to regulate the phospholipid or biological membrane binding activity of the protein.

AIIt is thought to participate in Ca^{2+}-dependent exocytosis [17, 23, 85], by bridging the secretory granules to the plasma membrane [85, 86], a property of AIIt that appears to be blocked by phosphorylation of the protein [87]. *In vitro,* AIIt displays several biological activities, all of which require Ca^{2+}. These biological properties include the binding and bundling of F-actin [74, 88–91], the binding and bridging of phospholipid vesicles [12, 92–94], the binding and bridging of biological membranes such as chromaffin granules [15, 95] and binding of heparin [87]. How the biological activities of AIIt contribute to the ability of AIIt to bridge secretory granules to plasma membrane is unknown. It is also unclear if the binding of AIIt to biological membranes involves the interaction of the protein with specific membrane receptors (AIIt binding proteins) or if the membrane binding activity of AIIt only involves the binding of AIIt to membrane phospholipids.

Many excellent reviews have been written on the general properties and regulation of the annexin family of Ca^{2+}-binding proteins. Therefore, the primary focus of this review is to evaluate the current literature dealing with the biochemistry of annexin II with special emphasis on the structure and function of AIIt.

Basic properties

Physical properties

Annexin II tetramer (AIIt) is composed of two copies of a 36-kDa heavy chain, called annexin II monomer (AIIm), and

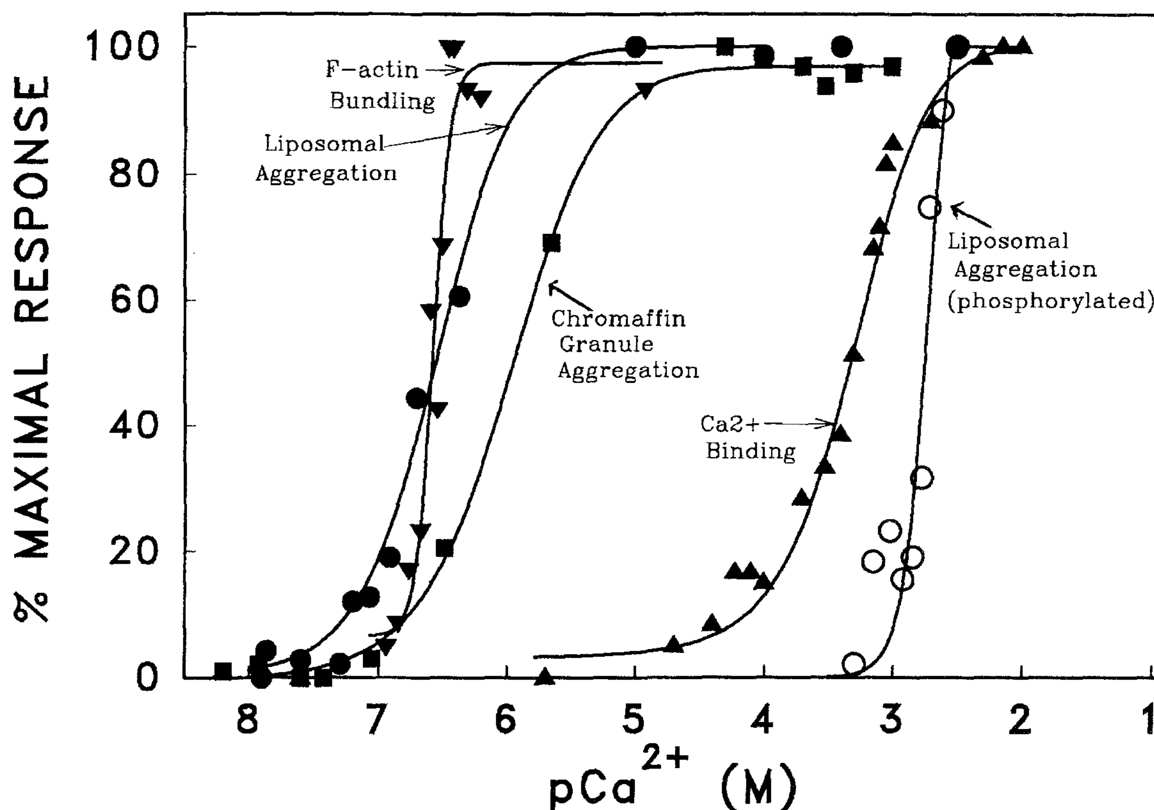


Fig. 2. Ca^{2+}-dependence of several biological activities of annexin II tetramer. Annexin II tetramer biological activity is expressed as % of maximal response. The conditions for measurement of the various biological activities has been described [94]. Phosphorylated refers to annexin II tetramer that was phosphorylated to about 2 mol phosphate/ mol AIIt by protein kinase C.

two copies of the 11 kDa light chain, p11 [74, 75]. The AIIm heavy chain consists of two functional domains. The amino-terminal domain contains the first 30 amino acids of the amino-terminus of the heavy chain and includes both the serine and tyrosine phosphorylation sites [96–100] and the binding site for the p11 light chain [101–103]. The remaining carboxyl domain, comprises the sites for Ca^{2+}, phospholipid [93, 103] and F-actin binding [91, 104] (Fig. 1). The amino-terminal domain and carboxyl-terminal domain of annexin II can be separated proteolytically. The limited proteolysis of either AIIm or AIIt by chymotrypsin, cleaves the protein into a 3-kDa amino terminus and a protease-resistant 33-kDa carboxyl-terminal core [98, 103].

The amino-terminal domain of annexin II plays an important regulatory role. AIIm aggregates chromaffin granules with a Kd (Ca^{2+}) of about 1 mM. Partial proteolysis results in the removal of the first 27 or 43 residues of the amino-terminus and reduces the Kd (Ca^{2+}) for aggregation of chromaffin granules from 141–28 μM Ca^{2+} respectively. Furthermore, the binding of the p11 light chain by AIIm results in the formation of the tetramer and reduces the Kd (Ca^{2+}) of chromaffin granule aggregation to about 2 μM [105]. Consistent with the dramatic differences in the Kd (Ca^{2+}) reported for the aggregation of biological membranes by AIIm and AIIt, the phospholipid aggregation properties of these proteins also appears to be very different. Several laboratories have reported that AIIt, but not AIIm aggregates phospholipid liposomes at submicromolar Ca^{2+} [94, 106]. Furthermore, in contrast to AIIt, AIIm cannot bundle F-actin at micromolar Ca^{2+} [107]. These results suggest that the amino-terminus exerts an inhibitory constraint on the chromaffin granule aggregation activity and F-actin bundling activity of the protein. The binding of the p11 light chain therefore appears to reverse this inhibitory restraint.

The carboxyl-terminal core has been shown to contain the sites for Ca^{2+}-binding, binding of acidic phospholipid [93, 103] and F-actin binding [91, 104]. The carboxyl-terminal core, as well as AIIm and AIIt bind Ca^{2+} with a Kd of about 0.5 mM Ca^{2+} [108, 109]. However, in the presence of acidic phospholipid, the carboxyl-core of annexin II as well as AIIm and AIIt exhibit micromolar Kd (Ca^{2+}) for Ca^{2+} binding [93] (Fig. 2). The observation that the carboxyl-core displays the same Ca^{2+}-binding affinity, stoichiometry and phospholipid dependency as intact AIIm [93, 106] suggests that this domain

is involved in modulating the phospholipid-dependent activation of Ca^{2+}-binding by annexin II. The last 16 residues of the carboxyl-terminus of the protein also contain a domain which has been shown to be conserved among 14-3-3 proteins [81]. This amino acid sequence has been suggested to be involved in mediating the interaction of a number of membrane-associated signal transduction proteins including SRC and RAF kinases.

Perhaps one of the most important questions to be asked is whether or not AIIm and AIIt have distinct physiological functions. This question can be partially answered by comparing the biochemical properties of these proteins. One of the basic biochemical properties exhibited by annexin II is the phospholipid-dependent enhancement of Ca^{2+}-binding. AIIm and AIIt have similar phospholipid requirements for this enhanced Ca^{2+}-binding; phosphatidylserine, phosphatidylinositol and phosphatidic acid support high affinity Ca^{2+}-binding but phosphatidylethanolamine and phosphatidylcholine do not [106, 110]. However, the Kd (Ca^{2+}) for binding to phosphatidylserine liposomes was reported to be < 10 nM for AIIt and about 2 µM for AIIm [106]. Furthermore, it was shown that addition of the p11 light chain to AIIm resulted in binding to phosphatidylserine liposomes with a Kd (Ca^{2+}) equivalent to that of AIIt. AIIt and AIIm have been shown to undergo Ca^{2+}-dependent conformational changes including a decrease in alpha-helical content and a shift in the emission maximum from 342–334 nm [108]. This shift in emmision maximum has been shown to be due to the movement of Trp-212 to a more hydrophobic environment in the presence of Ca^{2+}. Comparative analysis of the fluorescence characteristics of AIIm and AIIt indicates that the Trp is more deeply imbedded in the hydrophobic core of AIIm than AIIt [109]. Although both AIIm and AIIt possess low affinity Ca^{2+}-binding sites (Kd 0.5 mM), phospholipid binding results in a thousand fold decrease in the Kd (Ca^{2+}) for this conformational change for both proteins (AIIm, Kd 0.5 µM; AIIt, Kd 1.3 µM; Fig. 2). Direct analysis of the Ca^{2+}-binding stoichiometry of AIIm bound to phospholipid vesicles has revealed that AIIm bound about 10 mol Ca^{2+} at 50 µM Ca^{2+} [111]. In contrast, AIIt bound 15 mol Ca^{2+}, at this Ca^{2+} concentration suggesting that the Ca^{2+} stoichiometry was not related to the number of potential sites on the protein. AIIm and AIIt were also reported to bind to the phospholipid liposomes with a similar very high binding affinity of about 1 nanomolar. Therefore, analysis of the biochemical properties of AIIm and AIIt suggest that the most pronounced difference between AIIm and AIIt is the inability of AIIm to aggregate biological membranes, such as chromaffin granules at micromolar Ca^{2+} concentration and also to bundle F-actin at micromolar Ca^{2+} concentration.

AIIm and AIIt have been shown to be exist in distinct locations in the cell. Whereas the AIIm is distributed throughout the cell [112, 113], the association of the light chain with AIIm appears to mediate the interaction of AIIt with the plasma membrane [79]. Immunohistochemical localization studies have also confirmed the specific localization of AIIt to the plasma membrane in a variety of cells [85, 112–121]. Thus, the formation of AIIt determines the specific cellular localization of this protein. Therefore, the biochemical and histochemical studies suggest that AIIm and AIIt may have distinct physiological functions.

Molecular structure

The basic design of all members of the annexin family of Ca^{2+}-binding proteins includes a common motif consisting of a stretch of about 70 amino acids which is repeated 4 times or, in the case of annexin VI, 8 times. This motif is highly conserved among annexins and most of the amino acid variations in this region are conservative substitutions. In contrast, all annexins have a highly variable amino-terminal domain that is variable in length and composition and is believed to determine individual annexin functions. The crystal structure of annexin II has not been elucidated. Crystal structures of annexin V and annexin I have been reported [122–130]. Considering the homology between these annexins, these studies have allowed a reasonable prediction of the general structure of annexin II. The structure of AIIm is predicted to be composed of 4 domains of similar structure. Each domain contains 5 alpha-helices, denoted A–E, wound into a right-handed superhelix. Within the domain each of the alpha-helices is connected by a short stretch of amino acids comprising an interhelical loop such that two parallel helix-loop-helix substructures are formed (A-loop-B and D-loop-E) and these structures are stabilized by interhelical contacts.

Table 1. Ca^{2+}-binding sites of annexin II monomer

Type II sites	
	* * * *
	(1 17) (161)
Domain II	M - K - G - L - G - T - - - - - - - - D
	(201) (246)
Domain III	G - V - K - R- K - G - T - - - - - - E
	(277) (321)
Domain IV	M - K - G - K - G - T - - - - - - - - D
Type III sites	
	* * *
	(49) (52)
Domain I	G - V - D - E -
	* * *
	(87) (95)
	K - S - A - L - - - - E

The amino acid sequence of the Ca^{2+}- biding sites of human annexin II is depicted in the one-letter code. *, amino acid residues contributing a ligand for Ca^{2+} coordination.

Helix C appears to cross over the top of the domain and stabilize the helix-loop-helix structure. Domains I and IV and domains II and III are coupled by hydrophobic contacts. The 4 annexin domains form a planar cyclic arrangement in which the amino-terminal and carboxyl-domains interact to hold domains I and IV together. Overall, the annexin molecule is planar and curved with opposing concave and convex surfaces. The convex surface is thought to lie along the plane of the phospholipid membrane and to also contain the interhelical loops. The concave face of the protein faces the cytosol and contains both the amino-terminal domain and carboxyl-terminal domain as well as the stabilizing C helix.

The convex face of the protein also contains the Ca^{2+}-binding sites. Two novel types of Ca^{2+}-binding sites, which differ from the well-characterized EF-hand, have been identified in annexin I and annexin V crystals [122–124]. These studies along with site-directed mutagenic analysis of AIIm [131, 132] have allowed construction of a model of the Ca^{2+}-binding sites of AIIm (Table 1). Two distinct types of Ca^{2+}-binding sites have been shown to exist in AIIm. These include both type II and type III Ca^{2+}-binding sites. The type II Ca^{2+}-binding sites of AIIm are formed from a loop which connects the first and second alpha-helices (the A, B alpha-helices) of the second, third and fourth domain of the protein. An acidic residue, located about 38 residues downstream, between the fourth and fifth alpha-helices also comprises part of this Ca^{2+}-binding site. Ca^{2+}-coordination is accomplished by 3 peptide carbonyl oxygen ligands from alternate loop residues in the loop sequence and by bidentate carboxylate oxygens from the distant acidic residue. Water molecules are believed to provide 2 of the 7 ligands which collectively form a pentagonal bipyramid. In contrast, the type III Ca^{2+}-binding sites are formed from 3 ligand-donating residues: two peptide carbonyl oxygens from residues which form the loop between the fourth and fifth alpha-helices (the D, E alpha helices) and a bidentate carboxylate group from the fifth alpha-helix. Three water-molecule ligands contribute to the coordination of Ca^{2+} at this site. Three type III sites have been localized to domain I and domain IV of annexin I and at least two of these sites exist in domain I of AIIm.

Site-directed mutagenesis has allowed analysis of the effects of inactivation of either the type II or type III Ca^{2+}-binding sites on the biological activity of the protein. The Ca^{2+} concentration needed for half-maximal phosphatidylserine binding was shown to be 5–10 μM for an AIIm derivative with intact type II sites but defective type III sites whereas a mutant protein containing defective type II but intact type III sites required 200–300 μM Ca^{2+} for phosphatidylserine binding [131]. It was also shown that intact type II sites were required for association of the protein with the cytoskeleton, *in vivo* whereas if only the type III sites were intact, the mutant protein was cytosolic.

Distribution and localization

The tissue content of annexin II has been reported in both avian and mammalian tissues [104, 115, 133–135]. The protein is not detectable in heart, smooth muscle, skeletal muscle, liver, platelets and erythrocytes. Low concentrations of the protein have been reported in brain, while intermediate concentrations have been reported in spleen, kidney and adrenal gland. High concentrations have been reported in lung, placenta and intestine. Cell types that have been reported to contain intermediate or high concentrations of annexin II include the epithelial cells of skin, respiratory tract and intestine, endothelial cells of blood cells and chrondocytes of cartilage and connective tissue. Other cell types rich in annexin II include fibroblasts, macrophages, splenocytes and HeLa cells.

Annexin II has also been shown to exhibit a transient expression during maturation and differentiation of various cell types [136–138]. For example, the protein is present in myoblasts and myotubes but not in adult muscle. Although the protein is present at low concentration in the reactive astrocytes, ependymocytes and meningothelium of adult brain, the fetal brain contains much higher concentrations of annexin II than the adult brain. Furthermore, although annexin II is not detected in neurons, the concentration of the protein increases in many neuroblastoma. Annexin II is present at low concentration in F6 tetracarcinoma cells. However, activation of cellular differentiation causes a dramatic increase in the annexin II concentration in these cells [60]. It has also been shown that the differentiation of avian embryonic mesenchymal cells into cartilage and connective tissue results in a large increase in annexin II [139]. The tissue content of annexin II has also been shown to vary dramatically during pancreatic development [140]. Annexin II is expressed at very low concentration in pancreatic islets from 1 week old rats, but by 4 weeks of development the annexin II concentration has increased to moderate levels and by the adult stage the pancreatic levels are quite high.

Recently, Hatase and coworkers [141] examined the distribution of annexin II in rat liver. They found that although annexin II was absent from normal liver tissue, the activation of hepatocyte proliferation, which occurred after carbon tetrachloride-induced liver damage, resulted in the appearance of this protein in the liver hepatocytes. Similar increases in hepatocyte annexin II were observed after activation of liver regeneration due to partial hepatectomy. They concluded that activation of hepatocyte proliferation resulted in cellular increase in annexin II.

The cellular content of annexin II has also been shown to change during the cell cycle [80]. As cells divide and enter G1 phase there is a general decrease in the annexin II concentration. New synthesis of annexin II occurs as the cells enter S phase, however as the cells progress through S phase

there is a general reduction in the protein.

The relative tissue content of AIIt and AIIm can vary widely. Thymus contains almost exclusively AIIm whereas the AIIt/AIIm ratio varies from 50% in fibroblasts to almost 100% in intestinal epithelium. It has also been shown that the expression of p11 and AIIm mRNA is not always coordinated [142]. For example, F9 tetracarcinoma cells contain a high concentration of p11 whereas stimulation of the differentiation of this cell results in a large increase in AII and formation of AIIt [60].

Biological activities

Binding and bridging of membranes

The Ca^{2+}-dependent binding of the annexins to phospholipid liposomes is one of the fundamental properties of these proteins. However, both the Kd (Ca^{2+}) and the actual phospholipid specificity and affinity varies among the different annexins [93, 104, 110, 111, 143–147]. For example, annexin I binds to phosphatidylserine liposomes with a lower Kd (Ca^{2+}) than annexin V but the affinity of annexin V for phospholipid liposomes is much higher than that of annexin I. Generally, the annexins have been found to bind to phosphatidic acid, phosphatidylserine and phosphatidylinositol liposomes with increasing Kd (Ca^{2+}) [110]. Studies with chimeric annexins have suggested that the first annexin repeat (domain I, Fig. 1) may modulate the overall affinity for phospholipid binding but is probably not involved in determining the Ca^{2+} requirement for phospholipid binding [143]. The dramatic shift in the Kd (Ca^{2+}) of various annexins upon phospholipid liposome binding (Fig. 2) has led to the suggestion that the carbonyl oxygens of the phosphoryl headgroups of the phospholipid displace two coordinating water molecules and thereby participate in the coordination of Ca^{2+} within the Ca^{2+} binding site [128]. It has also been postulated that conserved basic residues located near the Ca^{2+} sites may contribute to the preference of the annexins for acidic phospholipid [125].

The exact nature of the interaction between the annexins and the membrane is unclear. The preference of the annexins to bind to acidic phospholipids and the observed reversibility of their Ca^{2+}-dependent membrane binding by Ca^{2+} chelators has suggested that these proteins bind to the membrane surface and do not penetrate the membrane. The observation that annexin I, annexin V, annexin VI and annexin VII possess voltage-gated Ca^{2+} channel activity [25, 27, 28, 148–150] has raised the possibility that the annexins may insert into the phospholipid bilayer. In fact, several annexin isoforms have been reported to be resistant to Ca^{2+} chelators but could be solubilized with detergent [124, 125, 129]. Analysis of the crystallographic structure of annexin V and annexin I [124, 125, 129] has revealed that these annexins possess some similarity to other ion channels. The 4 domains of these proteins form 4 superhelices which are folded into a near planar array presenting a hole in its centre which is coated with charged residues. Furthermore, the central hydrophillic pore and surrounding 4-helix bundles in the annexin molecule are oriented perpendicular to the membrane bilayer.

Several structural features of the annexins are inconsistent with a membrane-penetrating role for the protein. For example, the protein contains a high density of charged and polar groups where membrane insertion is expected to occur. Furthermore, the annexin helices that are suggested to span the membrane are, on average, 6–8 residues shorter than required for known membrane-spanning helices. Furthermore, the pseudo 2-fold axis of symmetry, which has been suggested to play a role in channel function, is obstructed by salt bridges. The alternative suggestion has been presented that the annexins may bind to the membrane surface resulting in disorder on both sides of the membrane, leading to increased ion permeability [11].

Recently, my laboratory used Ca^{2+}-loaded chromaffin granules as a model biological system, to examine the Ca^{2+} channel activity of annexin I–VI [29]. We found that only annexin V and annexin VI could cause the release of Ca^{2+} from the Ca^{2+}-loaded chromaffin granules. In the case of annexin VI, the annexin VI-induced release of Ca^{2+} was rapid and corresponded to 40% of total sequestered Ca^{2+}. Annexin VI-induced Ca^{2+} release occurred at extravesicular Ca^{2+} concentrations ranging from pCa^{2+} of 4.12–6.86. These results therefore suggested that ion-channel activity may not be a universal property of all annexin proteins.

Annexin II has been shown to bind to phospholipid liposomes with the lowest Kd (Ca^{2+}) of all the members of the annexin family [110]. In contrast to the universal property of phospholipid liposomal binding exhibited by all members of the annexin family, only 3 annexins have been shown to aggregate phospholipid liposomes, namely annexin I, annexin II and annexin VII. Of these 3 annexins only AIIt is capable of the aggregation of the phospholipid liposomes at sub-micromolar Ca^{2+} concentrations [94, 106]. Surprisingly, the phosphorylation of AIIt by protein kinase C results in inhibition of the phospholipid liposomal aggregation activity of the protein, without effecting the binding of the protein to the phospholipid liposomes [94] (Fig. 2). This suggests that although the membrane bridging sites reside in the carboxyl domain of AIIt, the amino-terminal domain can interact with and regulate these carboxyl domain sites. In contrast, the amino-terminal domain cannot regulate the phospholipid binding sites of the protein.

Although all annexins examined thus far are capable of the Ca^{2+}-dependent binding to biological membranes, such as chromaffin granules [15], the aggregation of chromaffin

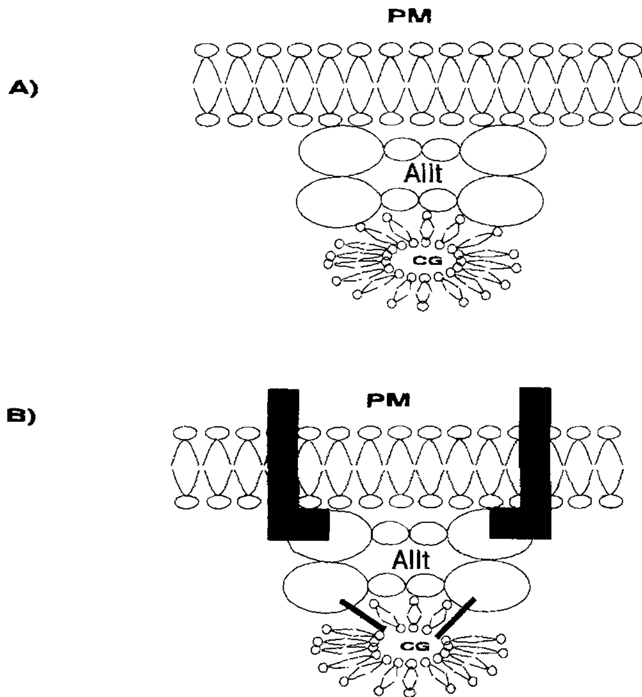


Fig. 3. Proposed mechanisms for membrane bridging activity of AIIt. The bridging activity of AIIt may involve the interaction of AIIt with membrane phospholipids (A) or both membrane phospholipids and membrane proteins (AIIt-binding proteins) (B). Furthermore, results from this laboratory [87, 95] suggest that the membrane-bridging activity of AIIt is due to the formation of a AIIt-AIIt bridge and not due to a single AIIt binding two different membranes. Abbreviations: CG = chromaffin granules; PM = plasma membrane.

granules at low micromolar Ca^{2+} concentrations appears to be a unique property of AIIt [95, 105, 151]. In order to understand the mechanism of AIIt-dependent bridging of biological membranes, two important questions remain to be answered. Firstly, it will be important to establish if the bridging of biological membranes by AIIt involves the binding of a single AIIt to two membranes, or if membranes are bridged through interactions between two annexins, each attached to a different membrane. Second, it will be important to establish if the interaction of AIIt with biological membranes is due to only the binding of the protein to the phospholipid bilayer or if the interaction of AIIt with biological membranes involves the binding of AIIt to both membrane phospholipids and membrane-associated AIIt binding proteins (receptors) (Fig. 3).

Experimental evidence has been presented to suggest that a single layer of bound annexin is sufficient for the bridging of phospholipid liposomes [145, 152]. Furthermore, it has been suggested that the bridging of biological membranes might involve a single layer of annexin at high Ca^{2+} concentrations and an annexin bridge at low Ca^{2+} concentrations [151]. My laboratory has demonstrated that the binding of AIIt to chromaffin granules can be experimentally dissociated from the AIIt-dependent bridging of these membranes *in vitro* [95]. We found that when salt was omitted from the reaction media, maximal binding of AIIt to chromaffin granules was observed, however, under these conditions AIIt-dependent chromaffin granule aggregation was not measurable. As the salt concentration was increased, AIIt-dependent chromaffin granule aggregation increased to a maximum, while AIIt binding to chromaffin granules decreased. Further increases in the salt concentration resulted in a decrease in both AIIt-dependent chromaffin granule aggregation and AIIt binding to granules. We therefore proposed that the AIIt-dependent aggregation of granules required two distinct conformations of AIIt. The first conformation (conformation A) required the presence of Ca^{2+} and the generation of conformation A allowed the binding of AIIt to the chromaffin granules. However, aggregation of the granules was not supported by conformation A. The observed inhibition of AIIt binding to chromaffin granules upon addition of salt could be explained, within this hypothesis, as an inhibitory effect of salt concentrations on the generation of conformation A. Mechanistically, it appeared that increasing salt concentrations increased the Ca^{2+} requirement for generation of conformation A [95]. When AIIt changed to conformation B, chromaffin granule binding activity was retained and chromaffin granule aggregation activity of the protein was promoted. The second conformation, conformation B, required the presence of an optimal concentration of salt. The Ca^{2+}-dependency of generation of conformation B on salt concentration appeared to be biphasic and varied from about 160 μM in the absence of added salt to about 0.9 μM in the presence of optimal salt concentration to 40 μM at 150 mM KCl. The observation that the chromaffin granule aggregation and binding activity of AIIt can be both activated and inhibited by cycles of Ca^{2+} and EGTA [95] suggests that the generation of conformation A and B was fully reversible.

The simplest mechanism to explain the chromaffin granule aggregation activity of conformation B, is that the protein, when in conformation B, either possesses two membrane binding sites, or a single membrane binding site and a site for an AIIt-AIIt bridge. In contrast, conformation A possesses only a single membrane binding site. However, if the mechanism of bridging of chromaffin granules by AIIt, involved two distinct membrane binding sites on a single AIIt molecule, one would predict that the loss of chromaffin granule aggregation activity at low salt concentration would be due to a salt requirement for the activation of the second membrane binding domain of AIIt. This model would therefore predict that at low salt concentration the salt-requiring membrane binding site of AIIt would be inactive and therefore the membrane binding stoichiometry of AIIt would be decreased. Since membrane binding stoichiometry of AIIt is maximal at low salt concentration, this model is not consistent with our

experimental data. Furthermore, we have also shown that the phosphorylation of AIIt, by protein kinase C or pp60^{c-src} inhibits the ability of the protein to bridge membranes but does not affect membrane binding by the protein [87, 88]. We therefore suggested that aggregation of chromaffin granules by AIIt could be caused by the interaction of AIIt molecules that were bound to the chromaffin granules and that this AIIt-AIIt interaction was modulated by the salt concentration and protein phosphorylation (Fig. 3).

Little is known about the domains of AIIt involved in the interaction of AIIt with chromaffin granule membranes or plasma membranes. It is unclear if the binding of biological membranes by AIIt is mediated by the phospholipid binding domains of the protein or requires both phospholipid lipid binding domains and domains capable of binding membrane proteins. However, considering that the binding of AIIt to these biological membranes is maximal at less than 25 μM Ca^{2+}, compared to the substantially higher Ca^{2+} requirement for F-actin binding and heparin binding, it is reasonable to suspect that the domain(s) of AIIt responsible for binding to membranes are distinct from the F-actin and heparin binding domains of AIIt. This speculation is further supported by our observatlon that tyrosine phosphorylation of AIIt inhibits both heparin binding and F-actin binding but not the binding of AIIt to biological membranes [87].

It is at present unclear if the interaction of AIIt with biological membranes is due to only the binding of the protein to the phospholipid bilayer or if the interaction of AIIt with biological membranes involves the binding of AIIt to both membrane phospholipids and membrane-associated AIIt binding proteins (receptors). Several experimental observations have suggested that the interaction of AIIt with plasma membranes cannot be explained purely by the phospholipid binding activity of the protein. Several laboratories have examined the interaction of AIIt with both phospholipid liposomes [106, 110, 145] and chromaffin granule membranes [95, 105, 147] and shown that the interaction of AIIt with either of these structures is Ca^{2+}-dependent and reversible by removal of Ca^{2+}. Furthermore, AIIt binding to these structures is reversed by 0.5 M NaCl. In contrast, it has been shown that the AIIt bound to the extracellular face of the plasma membrane of RAW117 metastatic cells is not removed by a buffer containing both 3 M KCl and 8 mM EDTA [71]. However, when the AIIt, obtained by detergent extraction of the RAW117 cells, was allowed to bind to the extracellular face of the plasma membrane of endothelial cells, it was observed that extracellular AIIt was removed by either 0.5 M NaCl or 2 mM EDTA. These results suggest that the binding of AIIt to the extracellular face of the plasma membrane of RAW117 cells is atypical of the binding of AIIt to other biological membranes. The simplest explanation is that protein receptors exist on the extracellular surface of the plasma membrane of the RAW117 cells and that these receptors demonstrate Ca^{2+}- and salt-independent binding of AIIt. Secondly, the interaction of AIIt with the intracellular face of the plasma membrane has been shown to involve the binding of AIIt to the intrinsic membrane protein, caveolin [153], therefore suggesting that AIIt is capable of interaction with membrane proteins (Fig. 3).

F-actin binding and bundling

Although qualitative data suggested that AIIt bound to F-actin [74] and either bundled F-actin [74]) or fragmented F-actin [154], a physiological role for AIIt in the regulation of F-actin could only be postulated if the Kd of actin binding by AIIt was sufficiently low (reviewed in [107]). The report that AIIt bound F-actin at millimolar Ca^{2+} [74, 89, 104] tended to preclude a physiological role for AIIt with respect to regulation of actin function. Therefore, we reexamined the kinetics and mechanism of interaction of AIIt with F-actin [88]. AIIt bound to F-actin in the presence of calcium with high affinity and in a cooperative manner. Quantitative analysis of binding curves indicated an apparent Kd (AIIt) of 0.226 μM ± 0.153 (2 SD, n = 3), a stoichiometry of AIIt/actin of 1:1.9 and a Hill coefficient of 1.37 ± 0.14 (2 SD, n = 3). Large anisotropic bundles were visualized by electron microscopy under these conditions and quantitation of bundling by both low speed sedimentation and light scattering yielded apparent Kd values between 0.12 and 0.27 μM AIIt. Filament bundling was dependent upon calcium and the calcium sensitivity was increased by raising the molar ratio of AIIt/F-actin. At saturating levels of AIIt, apparent $K_{0.5}$ values of 0.1–2 μM Ca^{2+} were obtained. In contrast, AIIm bundled F-actin to a much lesser extent and at much higher concentrations than for AIIt. Bundling of F-actin by annexin I was not detected at molar ratios of AIIt to actin as high as 2.5 mol/mol (AIIt/actin). At 5–10 μM Ca^{2+} and saturating levels of AIIt, F-actin bundling progressed very rapidly with a $t_{0.5}$ of 6 sec. The process was quickly reversed by addition of excess EGTA and bundles could be reformed by addition of a second burst of 5–10 μM Ca^{2+}. Considering that AIIt binds Ca^{2+} with a Kd of about 0.5 mM [108, 109] our results suggested that the binding of AIIt to F-actin induces a conformational change in AIIt, resulting in a decreased Kd (Ca^{2+}). Thus, our data suggested that AIIt could rapidly regulate F-actin bundling in a calcium-dependent manner at physiologically relevant calcium levels. Subsequently, these results have been confirmed by other laboratories [90, 155].

AIIt shares some common features with other actin bundling proteins (reviewed in [107]). Half-maximal binding of AIIt occurred near 0.18 μM free protein which is in the range reported for other bundling proteins such as nonmuscle alpha-actinin (0.22 μM); fascin (0.53 μM); the 30 kDa bundling protein of Dictyostelium (0.10 μM); actin-binding protein

(0.5 μM); Adducin (0.28 μM); and human erythrocyte band 4.9 (0.06–0.28 μM). Maximal binding of AIIt occurred at 0.58 mol/mol actin suggesting that each AIIt binds about 1.5–2 actins. This value is comparable to binding maxima of other actin bundling proteins such as fimbrin (0.29–0.35 mol/mol); villin (0.42–0.5 mol/mol); and synapsin I (0.147–0.4 mol/mol). Our binding data also indicated that AIIt binds with slight positive cooperativity yielding a Hill coefficient near 1.4. Adducin also binds cooperatively to actin ($n_H = 2.1$) as does synapsin I ($n_H = 1.35$). Thus, AIIt binds with high affinity and a stoichiometry typical of known actin filament bundling proteins.

Recently my laboratory identified the region of AIIt involved in F-actin bundling. We constructed a synthetic nonapeptide, Val-Leu-Ile-Arg-Ile-Met-Val-Ser-Arg, corresponding to residues 286–294 of AIIt, and demonstrated that this peptide completely inhibited the Ca^{2+}-dependent bundling of F-actin by this protein [91]. The inhibitory effect of the nonapeptide required preincubation with F-actin and was reversed by addition of excess AIIt. Kinetic analysis suggested that the nonapeptide reduced the K(0.5) but not the V_{max} of F-actin bundling. In contrast, addition of excess nonapeptide to AIIt-bundled F-actin did not reverse F-actin bundle formation. Although the nonapeptide produced a dose-dependent inhibition of AIIt-dependent F-actin bundling, the binding of AIIt to F-actin was not affected. The F-actin bundling region of AIIt is homologous to the proposed F-actin bundling site of myosin. Suzuki *et al.* [156] have shown that a heptapeptide (Ile-Arg-Ile-Cys-Arg-Lys-Gly) to the putative F-actin binding site of myosin inhibited the acto-S-1 (myosin subfragment 1) ATPase activity without effecting the binding of S-1 to F-actin. The observed inhibition of AIIt-dependent F-actin bundling activity by the nonapeptide, Val-Leu-Ile-Arg-Ile-Met-Val-Ser-Arg, presents the possibility that a similar site in F-actin may be responsible for both the activation of F-actin bundling activity of AIIt and for activation of myosin ATPase activity.

Perhaps one of the more interesting findings of our study [91] was that the nonapeptide inhibited AIIt-dependent F-actin bundling activity without causing dissociation of AIIt from F-actin. This suggested that the amino acid residues of AIIt involved in F-actin bundling are distinct from those involved in expression of F-actin binding activity. Clearly, additional factors must be involved in generation of F-actin bundling activity since both AIIm and AIIt contain identical amino acid sequences but AIIm is capable of only weak F-actin bundling activity [157]. The major difference between these proteins is that AIIt contains two copies of the heavy chain. The simplest explanation for the bundling activity of the tetramer and poor bundling activity of the monomer, is that the tetramer contains two F-actin binding sites, one on each of the two heavy chains. Theoretically, the binding of two distinct F-actin filaments by each heavy chain could result in the formation of F-actin bundles. However, if this suggestion was correct one would expect that the nonapeptide-dependent antagonism of F-actin bundling by AIIt would also effect the affinity or stoichiometry of AIIt-dependent F-actin binding, which is not the case [91]. The observation that the nonapeptide inhibits F-actin bundling but not F-actin binding suggests that the two (or more) F-actin binding sites on each of the heavy chains of AIIt are not directly involved in the F-actin bundling activity of the protein. It is possible that once bound to F-actin, the interaction of the F-actin with the bundling site of AIIt causes a conformational change in the AIIt resulting in unmasking of additional F-actin binding site and that the interaction of these new binding sites with F-actin results in formation of F-actin bundles. However, one would expect that if this was correct that the nonapeptide would not only inhibit bundling activity of AIIt, but also inhibit F-actin binding by AIIt.

We have proposed that the ability of AIIt to bundle F-actin could be due to self-association of F-actin-bound AIIt [91]. Specifically, we proposed that once AIIt binds to F-actin, an interaction occurs between the F-actin bundling site of AIIt and F-actin which results in a conformational change in AIIt. As a result of this conformational change, and only as a result of this conformational change, AIIt acquires the ability to interact with other AIIt molecules which are bound to F-actin and therefore in the active conformation. This model would therefore predict that a peptide to the F-actin binding site would prevent the conformational change and therefore inhibit self-association of F-actin-bound AIIt, without effecting the affinity or stoichiometry of AIIt-dependent F-actin binding. The possibility exists that interaction of AIIm with the F-actin bundling site also results in the activated conformation, however the absence of quaternary structure of the monomer, reflected in decreased size or absence of light chains may prevent proper self-association of F-actin-bound monomer. This could therefore explain the poor F-actin bundling activity of the AIIm. Similarly, annexin-I contains an amino acid sequence homologous to the putative F-actin bundling region of AIIt. The corresponding region in annexin-I, Ala-Leu-Ile-Arg-Ile-Met-Val-Ser-Arg, differs from the AIIt sequence in only a single amino acid substitution, namely a Val in AIIt is substituted for Ala in annexin-I. Although annexin-I binds F-actin, this protein cannot bundle F-actin [157]. If it is assumed that after binding to F-actin, interaction of the annexin-I bundling site with F-actin results in activation of the protein, then it is reasonable to suspect that the protein cannot bundle F-actin either because of its size or because it lacks the p11 light chains.

The role that F-actin binding or bundling plays in the physiological function of the protein is unclear. Since the phosphorylation of AIIt inhibits F-actin binding but does not inhibit the binding of the protein to chromaffin granules, it would appear that F-actin binding does not play a role in the binding of AIIt to biological membranes [87, 94].

Heparin binding

Annexin-IV was originally demonstrated to possess carbohydrate binding activity [158]. This annexin was shown to bind to fetuin and heparin in a Ca^{2+}-dependent and specific manner. N-acetylneuraminic acid inhibited the binding of annexin-IV to the fetuin and heparin most strongly, however, other saccharides and asialoglycoproteins such as chrondroitin sulphate, hyuralonic acid and calf asialofetuin but not chitin sulphate or chrondroitin were also highly inhibitory, suggesting that annexin IV possesses broad carbohydrate binding specificities. It was also demonstrated that annexin IV bound to charged lipid-bound oligosaccharides, such as various gangliosides but did not bind to sulphatide [Gal(3-SO4)β1-1Cer].

Recently we examined AIIt for potential carbohydrate binding activity [87]. We found that AIIt binds to a heparin affinity column in the presence of millimolar Ca^{2+} and was eluted from the column by buffer containing EDTA. In contrast, when tyrosine-phosphorylated AIIt was applied to the heparin affinity column, the protein did not bind and was recovered in the column flow-through. Our results have suggested that the carbohydrate-binding activity of AIIt is inhibited by tyrosine phosphorylation. This presented the possibility that distinct domains of AIIt are involved in the binding to biological membranes and in the binding to heparin. Considering that the intracellular Ca^{2+} concentration never reaches millimolar concentrations, it is extremely unlikely that the carbohydrate binding property of AIIt contributes to the physiological function of the protein. However considering the recent reports describing the presence of AIIt on the outside of the cell [71] where the protein is thought to be involved in cell-cell adhesion, it is possible that the carbohydrate binding activity of AIIt may play a role in the physiological function of extracellular AIIt.

Regulation of annexin II tetramer by serine and tyrosine phosphorylation

As discussed above the AIIm heavy chain consists of two functional domains. The first, the amino-terminal domain, contains the first 30 amino acids of the amino-terminus of the heavy chain and incorporates the serine and tyrosine phosphorylation sites [96–100]. Activation of protein kinase C in AG1523 fibroblasts or MDBK kidney cells has been shown to result in the phosphorylation of AII at Ser-25 [99]. The *in vitro* phosphorylation of AIIt, by protein kinase C [31, 94, 99, 159] also occurs at Ser-25 [99] and has been shown to increase the Kd (Ca^{2+}) for aggregation of phospholipid liposomes, without affecting the phospholipid binding properties of the protein [94]. AIIt has also been shown to be phosphorylated *in vivo* by protein tyrosine kinases. For example, the expression of transforming protein tyrosine kinases in a variety of cells has been shown to correlate with the appearance of phosphotyrosine in AII [116, 160–162] and in many cells AII is a major *in vivo* substrate of $pp60^{v\text{-}src}$ [162–164]. AIIt is also a major *in vivo* substrate for the constitutive protein tyrosine kinase activity of bovine articular chondrocytes [165]. Activation of growth factor receptors, such as PDGF, has been shown to result in the tyrosine phosphorylation of AII [166–168]. The phosphorylation of AII in $pp60^{v\text{-}src}$ transformed cells or in cells activated by PDGF is identical to the site phosphorylated on the protein *in vitro* by $pp60^{v\text{-}src}$, namely tyrosine-23 [166].

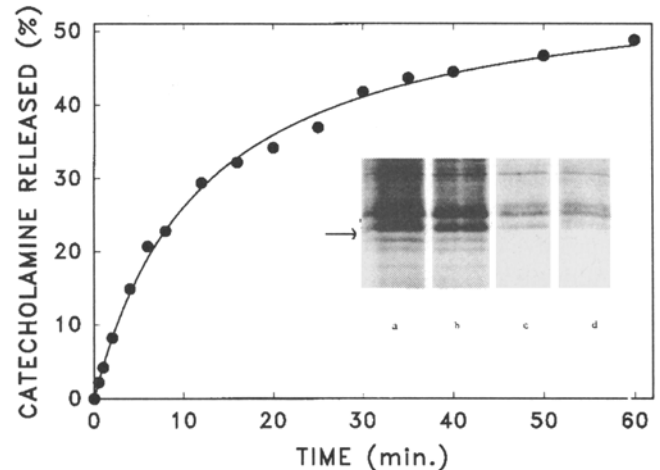


Fig. 4. Dephosphorylation of annexin II tetramer during cholinergic stimulation of adrenal chromaffin cells. Cultured bovine adrenal chromaffin cells were incubated with ^{3}H[norepinephrine] for 3 h. The cells were stimulated with 100 μM acetylcholine, aliquots removed at the times indicated, and analyzed for ^{3}H-[catecholamine]. Results are expressed as the percent of catecholamine released into the culture supernatant as a function of the total cellular content of ^{3}H-catecholamine. *Inset*: adrenal chromaffin cells were incubated in phosphate-free DMEM and ^{32}P [PO_4] (1 mCi/ml) for 16 h. The media was replaced with DMEM and the cells were unstimulated (a), or stimulated with 100 μM acetylcholine for 0.5 min (b), 5.0 min (c) or 30 min (d). Following lysis of the cells the annexin II monomer and annexin II tetramer were immunoprecipitated with a polyclonal antibody to the annexin II monomer and subjected to SDS page and autoradiography. The p36 heavy chain is shown by the arrow.

The stimulation of adrenal medulla cells has been shown to result in changes in the phosphorylation pattern of a variety of cellular proteins [169, 170], and activation of protein phosphatases have been suggested to produce a 48% inhibition of catecholamine release [171]. However, only a single report describes the phosphorylation of AIIt after cholinergic stimulation of cultured adrenal chromaffin cells [172]. These authors reported that AII (monomer and tetramer were immunoprecipitated) was phosphorylated on predominately alkali-sensitive sites (Ser and Thr) during cholinergic stimulation. Other reports have documented changes in protein

kinase activity in adrenal chromaffin cells and reported that upon cholinergic stimulation, the protein kinase C activity is increased [173–175] and the pp60^{src} activity is decreased [176]. Furthermore, it has been shown that inhibition of the activity of calmodulin-dependent protein kinases or protein kinase C are not required for Ca^{2+}-dependent secretion [174, 177], but PKC may play a modulator role [177].

We have reexamined the time course of phosphorylation of AIIt during the cholinergic stimulation of cultured adrenal chromaffin cells (Fig. 4). Our results have suggested that upon stimulation of chromaffin cells, AIIt is rapidly dephosphorylated. The dephosphorylation of AIIt corresponded with the release of catecholamine from the chromaffin cells.

We have also characterized the phosphorylation of annexin II tetramer (AIIt) by protein kinase C and pp60$^{c\text{-}src}$ *in vitro*. Protein kinase C catalyzed the incorporation of 2 mol phosphate/mol AIIt [94]. Phosphorylation of AIIt resulted in inhibition of the rate and extent of lipid vesicle aggregation without significantly affecting the binding of the phosphoprotein to the lipid vesicles. Phosphorylation of AIIt also increased the $A_{50\%}$ (Ca^{2+}) of lipid vesicle aggregation from 0.18–0.65 mM. We have also reported the phosphorylation of AIIt, *in vitro* to 0.89 mol phosphate/mol AIIt by pp60$^{c\text{-}src}$ [87] and the phosphorylation site was identified as Tyr-23. The native and phosphoprotein bound to chromaffin granules with similar affinity but unlike native protein, the phosphoprotein did not aggregate these granules. In contrast to the native protein, the phosphoprotein did not bind to F-actin nor did the phosphorylated protein bind to a heparin affinity column. Furthermore, the phosphorylation of AIIt by pp60$^{c\text{-}src}$ inhibited the *in vitro* ability of this annexin to form a complex consisting of plasma membrane, chromaffin granules and AIIt. In conclusion, work from my laboratory has suggested that the phosphorylation of AIIt is a negative modulator of AIIt activity and therefore, the activation of protein phosphatase activity, and dephosphorylation of AIIt, may be an important event for the activation of AIIt.

Clearly, elucidation of the mechanism of action of AIIt, in exocytosis, will await resolution of the question of whether or not AIIt is phosphorylated or dephosphorylated at the onset of exocytosis. A clear resolution of this question will require analysis of the patterns of phosphorylation of immuno-

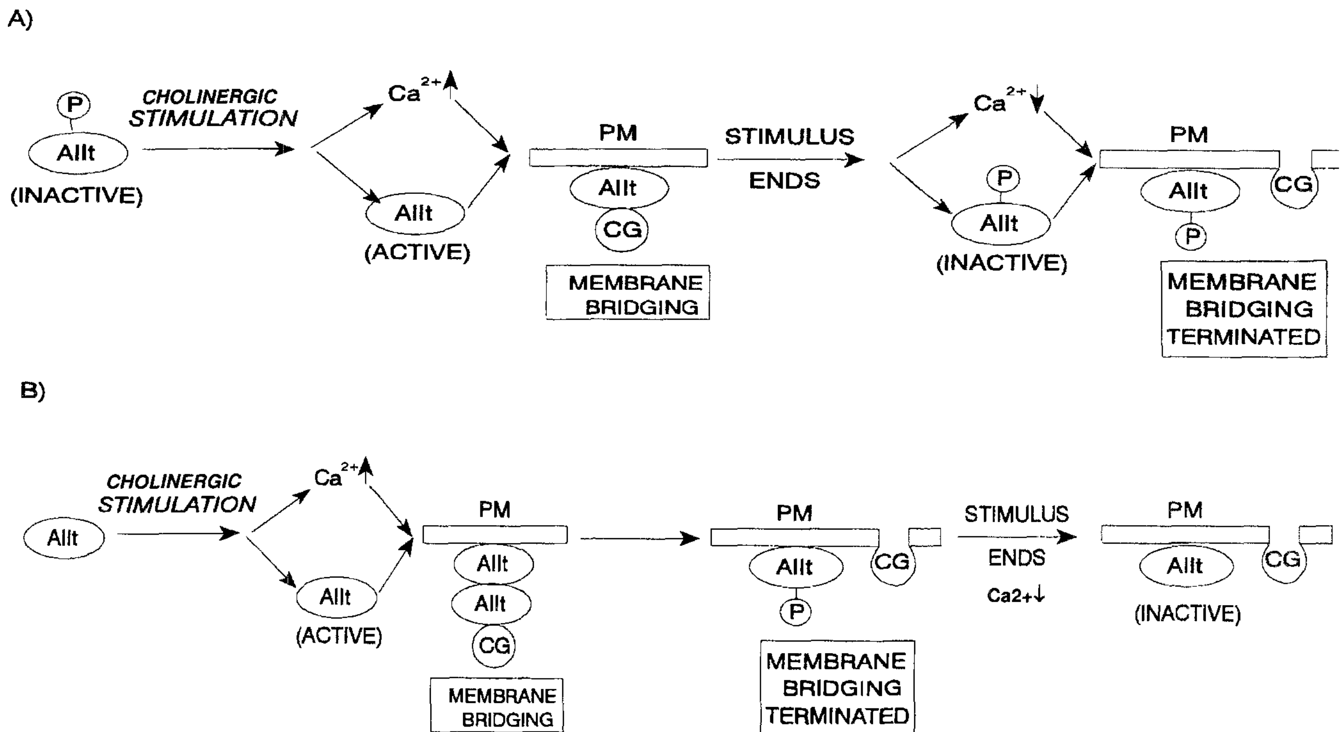


Fig. 5. Role of phosphorylation in the regulation of AIIt function. Models are proposed to explain how AIIt may participate in exocytosis in lieu of results suggesting that AIIt is either phosphorylated (A) or dephosphorylated (B) at the onset of cholinergic stimulation of bovine adrenal chromaffin cells. The basic assumption of the models is that the phosphorylation of AIIt blocks the membrane bridging activity of AIIt [87] and therefore chromaffin granules are not bound to the plasma membrane by the phosphoprotein. In model A, the cholinergic stimulation of adrenal chromaffin cells results in the dephosphorylation and activation of AIIt. Therefore, this model requires the presence of a AIIt-phosphatase which would be activated by cholinergicdependent increases in cytosolic Ca^{2+} Alternatively, a protein kinase, activated during cholinergic stimulation, could phosphorylate and activate the AIIt-phosphatase. In model B, cholinergic stimulation results in an increase in cytosolic Ca^{2+} which activates the membrane of AIIt. The phosphorylation of AIIt results in the inactivation and movement of AIIt from the plasma membrane-chromaffin granule complex. The movement of AIIt allows the fusion proteins to promote fusion of chromaffin granules with plasma membrane. Abbreviations: CG = chromaffin granules; PM = plasma membrane.

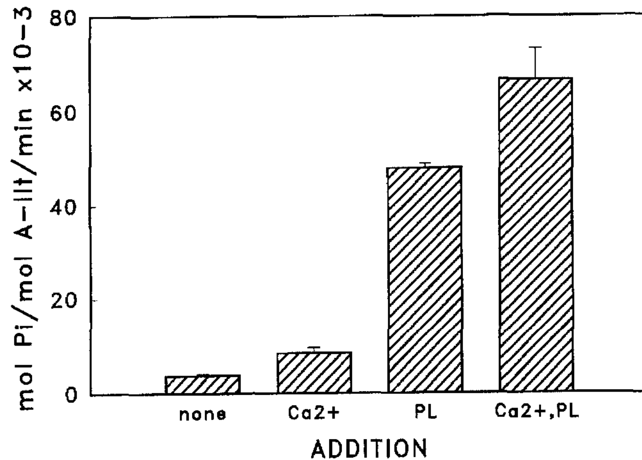


Fig. 6. Stimulation of tyrosine phosphorylation of annexin II by Ca^{2+} and phospholipid. Annexin-II tetramer (0.6 μM) was phosphorylated with recombinant pp60$^{c\text{-}scr}$ (43 nM) in buffer A (25 mM HEPES [7.5], 10 mM $MgCl_2$ and 0.5 mM EGTA) [NONE], or phospholipid and buffer A [PL], buffer A and 0.6 mM $CaCl_2$ [Ca^{2+}] or buffer A, 0.6 mM $CaCl_2$ and phospholipid [Ca^{2+}, PL]. The reaction was initiated by addition of 25 , μM ATP at 30°C for the times indicated. The phospholipid vesicles (100 μL/ml) were composed of 400 μg/ml PS and 40 μg/ml DAG.

precipitated AIIt. Since previous studies ([172] and Fig. 4) have used antibodies that immunoprecipitate the 36-kDa chain of both AIIm and AIIt, these studies have assumed that the phosphorylation patterns of AIIm and AIIt are similar. AIIm and AIIt may not share similar regulatory pathways. It has been shown that purified synaptic membranes contain serine and tyrosine kinases capable of the phosphorylation of AIIm and AIIt [90]. However, the phosphorylation of AIIm was shown to occur on serine residues while the phosphorylation of AIIt occurred on both serine and tyrosine residues. Therefore, analysis of the phosphorylation patterns of AIIt will require the use of an antibody to immunoprecipitate the p11 light chain of AIIt, followed by SDS PAGE analysis of the 36 kDa heavy chain. Figure 5 presents two models depicting the possible role of AIIt in exocytosis. The models illustrate the two possibilities of the phosphorylation or dephosphorylation of AIIt during the stimulation of secretion in adrenal chromaffin cells.

Recently, we examined the kinetics of phosphorylation of AIIt by pp60$^{c\text{-}src}$. As shown in Fig. 6, the initial rates of phosphorylation of AIIt are stimulated by the presence of Ca^{2+} and phospholipid liposomes. Surprisingly, the initial rate of phosphorylation of AIIt in the presence of Ca^{2+} was only about 12% of the rate of phosphorylation of AIIt in the presence of both Ca^{2+} and phospholipid. In contrast, the initial rates of pp60$^{c\text{-}src}$ activity are not influenced by the presence or absence of Ca^{2+} or phospholipid liposomes. Our results therefore suggest that AIIt bound to the membrane is a substrate of pp60$^{c\text{-}src}$. Since the binding of AIIm to the p11 light chain has been shown to direct AII to the plasma membrane *in vivo* [79], it is reasonable to suggest that only AIIt will be phosphorylated by protein kinases that either reside on the plasma membrane or are directed to the plasma membrane during cellular stimulation.

Regulation of DNA polymerase α by Annexin II

Recently two proteins, initially called C1 and C2 [178, 179] and more recently PRP-1 and PRP-2 [180] have been shown to form a heterodimer and in the heterodimeric form this complex functions to allow DNA polymerase α to utilize primer/templates with low primer:template ratios. The heterodimer, referred to as primer recognition protein (PRP) has been identified in HeLa cells [180, 181] monkey CV1 cells [178, 179] and human placenta [182]. Purified PRP from HeLa cells has been shown to be composed of two subunits of 36 kDa (PRP 1) and 41 kDa (PRP 2) [180]. Amino acid sequence analysis has suggested that PRP 1 is annexin II monomer [73] and that PRP 2 is the glycolytic enzyme, 3-phosphoglycerate kinase [182]. The PRP heterodimer increases the utilization of short primers and produces a greater than 20-fold decrease in the Km for template primers with low primer density, but does not affect the Km for template primers with high primer densities. This suggests that the PRP stimulation of polymerase α does not result from increased affinity for primer termini *per se* but from a decrease in the nonproductive binding of polymerase α to single-stranded DNA. It has therefore been suggested that PRP facilitates primer recognition by enabling polymerase α to slide along the template until it encounters a primer terminus. Alternatively, PRP could function to increase the rate of polymerase α recycling upon nonproductive binding to single-stranded DNA.

Using immunoblotting, immunofluorescence microscopy and immunoelectron microscopy of HeLa cells, hepatocytes and pancreatic tissue, Kumble and Vishwanatha [183] have demonstrated the presence of AIIm and DPG in the cell nucleus. In HeLa cells it was suggested that 6% of the total DPG was present in the nuclear extracts while 10% of the total AIIm was present in the nucleus. The concept of the cell nucleus containing glycolytic enzymes is not novel. Work from my laboratory [184, 185] has demonstrated the presence of enolase in the cell nucleus and specifically at the microtubule organizing centres.

The identification of annexin II as a growth regulated gene 1 B6 [53] has also presented the possibility that annexin II might play a role in DNA replication. Recently, Kumble *et al.* [78] exposed exponentially growing HeLa cells to antisense phosphorothioate oligodeoxynucleotides to annexin II

and DPG and it was observed that ongoing DNA synthesis was reduced. It was also shown [78] that exposure to the antisense nucleotides blocked progression from S phase to G2 phase of the cell cycle. Similarly, Vishwanatha and Kumble [77] showed that immunodepletion of Xenopus annexin II from cell-free extracts of Xenopus eggs resulted in a loss of DNA replicative ability. Replicative ability was restored to the immunodepleted extracts by the addition of purified human AIIm.

Role of annexin II tetramer in secretion

The possible involvement of AIIt in exocytosis was first postulated based on both the localization of AIIt to the plasma membrane [74, 75, 79, 112, 115–121, 186] and the ability of AIIt to aggregate isolated chromaffin granules [95, 105]. It was suggested that the *in vitro* chromaffin granule aggregation activity of AIIt might model the *in vivo* docking of chromaffin granules with the apical plasma membrane prior to secretion [105]. Furthermore, electron microscopic analysis has suggested that AIIt forms cross-links between secretory granules and plasma membrane [85, 86].

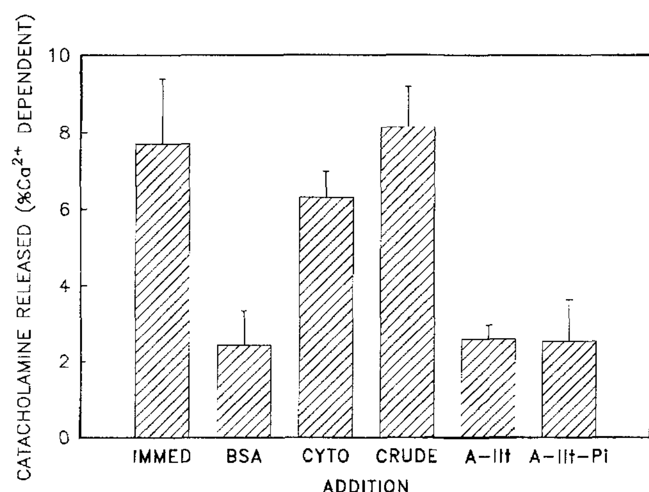
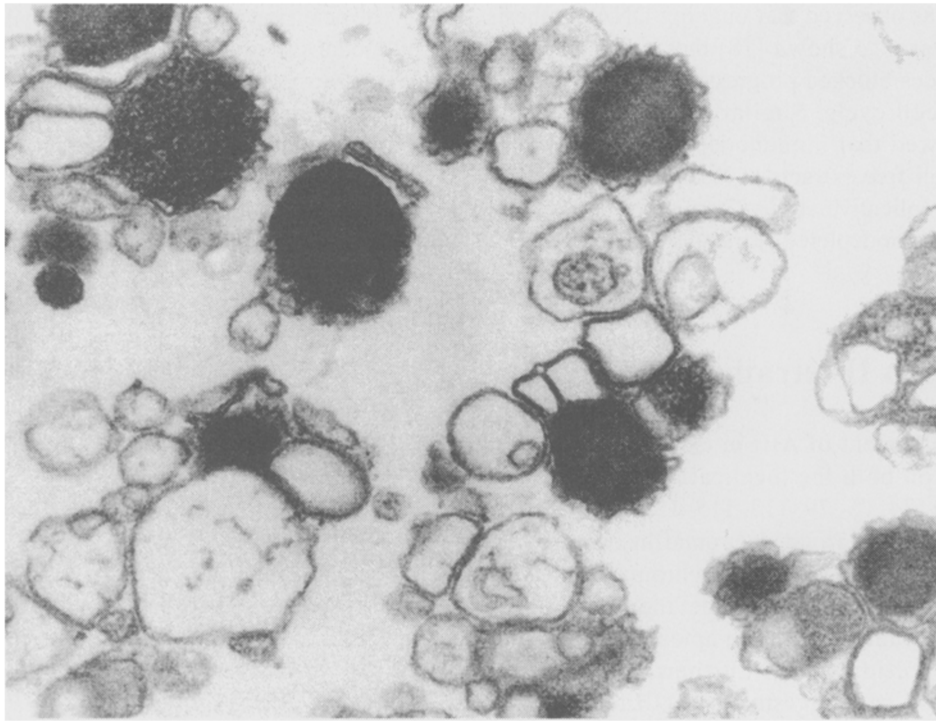


Fig. 7. Reconstitution of secretion in detergent permeabilized adrenal medulla cells. Cultured bovine adrenal medulla cells were incubated for 3 h with ^{3}H-[norepinephrine]. The cells were permeabilized with 10 μM digitonin for 6 min and immediately stimulated for 10 min with 20 μM Ca^{2+} (immedt) followed by determination of the percentage of total cellular ^{3}H-[catecholamine] released into the culture supernatant. Alternatively, cells were permeabilized then run-down by 20 min incubation with Ca^{2+}-free media containing 100 μg/ml bovine serum albumin (BSA) or 100 μg/ml adrenal chromaffin cell cytosol (CYTO) or 100 μg/ml of the crude annexin fraction, i.e. cytosolic proteins purified on the basis of Ca^{2+}-dependent binding to cellular membranes (CRUDE) or 10 μg/ml of annexin II tetramer which was purified from the crude annexin fraction (A-IIt) or purified annexin II tetramer that was phosphorylated to 2 mol phosphate/mol AIIt by protein kinase C (A-IIt-Pi).

The detergent permeabilized adrenal medulla cell is a useful model system for studying the role of cytosolic proteins in exocytosis [187–193]. When permeabilized adrenal medulla cells are incubated in Ca^{2+}-free buffer, cytosolic proteins are lost and the loss of these proteins correlates with the loss of the Ca^{2+}-stimulated catecholamine release [194]. In contrast, the incubation of the permeabilized cells, with Ca^{2+}-free buffer containing AIIt, has been shown to retard the loss of the secretory response [17–22, 81, 82]. These results are controversial and several laboratories have reported either that AIIt does not activate secretion [195, 196], or that AIIt-depleted cytosol activates secretion in permeabilized adrenal medulla cells [20]. Although AIIt can aggregate biological membranes, the protein does not fuse these membranes [105]. This suggests that in order for AIIt to activate secretion, fusogenic proteins remain associated with the plasma membrane during run-down.

My laboratory has presented preliminary evidence that the ability of AIIt to restore secretion may be due to a protein contaminant of the purified AIIt and that highly purified AIIt does not reconstitute secretion in permeabilized adrenal medulla cells (see [197] and Fig. 7). Specifically we found that when adrenal medulla cells were permeabilized with 10 μM digitonin and immediately stimulated with 20 μM Ca^{2+}, about 8% of total catecholamine was released. In contrast, when the cells were permeabilized, then run-down by a 10 min incubation with Ca^{2+}-free media containing bovine serum albumin, Ca^{2+}-dependent secretion was reduced to about 2.4%. Incubation of these cells, during run-down, with adrenal medulla cytosol or a partially purified fraction of AIIt, at protein concentrations identical to the bovine serum albumin, restored Ca^{2+}-dependent secretion to 6 and 8% of total catecholamine released, respectively. However addition of highly purified AIIt, during run-down, did not restore secretion (2.6% of total catecholamine released). The highly purified AIIt was not denatured during purification as the protein aggregated chromaffin granules with a $A_{50\%}$ (Ca^{2+}) of 2 μM. We have identified the point in our purification procedure in which the ability of AIIt to reconstitute secretion is lost. This corresponds with the loss of a low molecular weight protein contaminant from AIIt. Our current hypothesis is that AIIt and this contaminant protein are required to reconstitute secretion in the permeabilized adrenal chromaffin cells.

The docking and fusion of secretory granules with plasma membrane has been extensively studied in both yeast [198] and mammalian neurons [199–205]. A number of both integral and soluble proteins have been postulated to be involved in both the movement of secretory granules to the plasma membrane and the docking and fusion of the secretory granules with the plasma membrane exocytotic sites. For example, in nervous tissue, the plasma membrane associated proteins, SNAP-25 and syntaxin, have been suggested to form

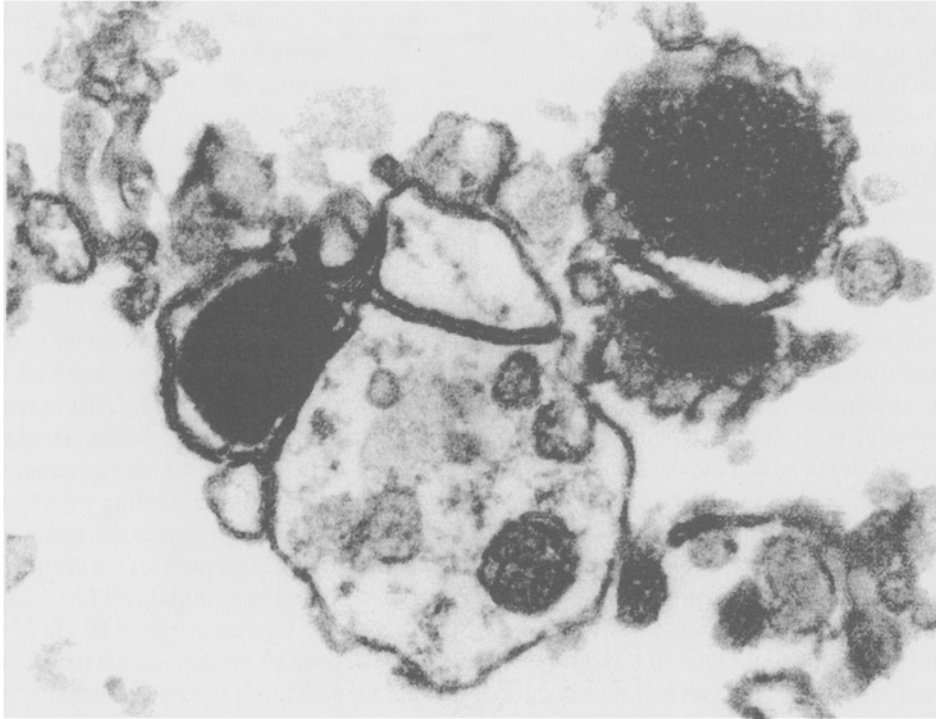


A

Fig. 8. Electron micrographic analysis of the interaction of annexin II tetramer with purified chromaffin granules and plasma membrane. Chromaffin granules (0.1 mg) were incubated in the presence of 1.23 μM AIIt and 0.21 mg of purified plasma membrane in a buffer containing 0.3 M sucrose, 25 mM HEPES (pH 7.5), 20 μM $CaCl_2$, 30 mM KCl for 15 min at 20°C. The samples were fixed by adding 50% gluteraldehyde to a final concentration of 2.5 % . After a 2 h incubation at 4°C samples were sedimented at 14,000 × g and the pellets were postfixed in 1% Osmium tetroxide. The samples were then dehydrated in acetone and embedded in Epon 812. Thin sections were stained with aqueous uranyl acetate and lead citrate and examined with a Hitachi H-7000 electron microscope. The vesicles with dark cores represent chromaffin granules. The translucent vesicles represent plasma membrane vesicles. A, magnification 60,000×; B, magnification 100,000×.

a fusion complex with the synaptic vesicle protein, synaptobrevin. The formation of this complex requires ATP and cytosolic proteins including the N-ethylmaleimide sensitive factor (NSF) the soluble NSF attachment proteins (SNAPs) and n-Secl. The formation of the fusion complex between SNAP-25, synaptobrevin, syntaxin, NSF and SNAP's is Ca^{2+}-independent and therefore it has been postulated that a Ca^{2+}-binding protein must be involved in the docking and fusion of the plasma membrane with the synaptic vesicles. However, the docking of the synaptic vesicles with the plasma membrane has also been suggested to involve the binding of the plasma membrane proteins, neurexin and syntaxin, with synaptotagmin, an integral Ca^{2+}-binding protein of the synaptic vesicle. Although a clear picture of the role of various proteins in secretory vesicle docking and fusion has not emerged, it is clear that the docking and fusion of secretory granules with the plasma membrane exocytotic sites will involve a cascade of protein-protein interactions, between soluble cytoplasmic proteins and proteins of the plasma membrane and synaptic vesicles. The experimental observations to date, are consistent with a model in which one set of Ca^{2+}-independent proteins are involved in the fusion of secretory granules with the plasma membrane and another set of Ca^{2+}-dependent proteins are involved in the docking of the secretory granules with the plasma membrane. Although present at very low concentration, SNAP-24, NSF, synaptotagmin, alpha-SNAP and syntaxin have been shown to be present in adrenal chromaffin cell [206], suggesting these proteins may play a role in the fusion of chromaffin granules with plasma membrane. Collectively, the experimental evidence suggests that AIIt may play the role of the Ca^{2+}-dependent docking protein.

AIIt is thought to participate in Ca^{2+}-dependent exocytosis [17, 23, 85], by bridging the secretory granules to the plasma membrane [85, 86]. Electron microscopic analysis of both acetylcholine activated cultured adrenal medulla cells and



B

Fig. 8 (continued).

stimulated anterior pituitary cells has suggested that AIIt forms cross-links between secretory granules and plasma membrane [85, 86]. However, the possibility that AIIt might form similar structures between plasma membrane and secretory granules, in a cell-free system, has not been investigated. We have found that when AIIt is incubated in the presence of Ca^{2+} with purified adrenal medulla plasma membranes (PM) and purified chromaffin granules (CG), that structures consisting of AIIt-bridged plasma membranes (PM-AIIt-PM), AIIt-bridged chromaffin granules (CG-AIIt-CG) and structures consisting of chromaffin granules bridged to plasma membrane (CG-AIIt-PM) are formed (Fig. 8). These complexes were not formed in the absence of AIIt or in the presence of AIIt if Ca^{2+} was omitted from the incubation media. Furthermore, when these structures are subjected to sucrose density centrifugation, the three types of AIIt bridged complexes can be resolved. We also found that although $pp60^{c\text{-}src}$ phosphorylated AIIt, bound to both plasma membrane and chromaffin granules, AIIt-bridged complexes were not formed [87]. These studies have suggested that AIIt can form CG-AIIt-PM complexes *in vitro* and that phosphorylation of AIIt blocks complex formation.

Role of annexin II tetramer in cell–cell interactions

The appearance of annexin on the extracellular face of the plasma membrane is unexpected since the annexin family of proteins lack a hydrophobic signal sequence that has been characterized in most secreted proteins. However, even though no mechanism for the extrusion of annexin proteins is known, there is convincing evidence from a number of independent laboratories to substantiate the presence of annexins on the outer face of the plasma membrane of intact cells. Annexin I has been shown to be selectively secreted from human prostate [207], whereas annexin-V has been shown to be present on the extracellular face of chondrocytes [63, 68] and annexin-VI has also been shown to be associated with the cell surface of mouse mammary epithelial cells [56]. Annexin II has been shown to be present on the surface of endothelial cells [208], skin keratinocytes [209] and several non-metastatic and metastatic tumour cells [70].

The extracellular presence of the annexins when considered with the ability of annexin-II to bind to phospholipid membranes, and components of the extracellular matrix such

as actin and collagen [210] suggests a possible role for these proteins as cell-cell or cell-extracellular matrix adhesion agents. Indeed, there is evidence that annexin-II, in association with other cell-cell and cell-extracellular matrix adhesion factors, is responsible for maintaining Ca^{2+}-dependent sponge cell aggregation [211]. Specifically, it was shown that annexin-II directly promoted the aggregation of sponge cells and that an antibody to annexin-II significantly blocked cellular aggregation. The high (80%) amino acid sequence similarity reported between sponge and vertebrate annexin-II [211] suggests a conserved function of this protein.

The insidious property which distinguishes metastatic tumour cells from non-cancerous cells is their ability to migrate from their original site of proliferation, via the circulatory system, to a foreign host organ which they colonize prior to proliferation. Recent studies have suggested that one of the key events that allow metastatic cells to bind to their target organ is the expression, on the outside of the metastatic cell, of certain adhesion proteins [212, 213]. A number of different cell adhesion molecules such as cadherins, selectins and integrins have been shown to be involved in tumour metastatic processes (e.g. [214]). Recently, several non-integrin tumour cell surface proteins associated with divalent cation-dependent adhesion of murine RAW117 large-cell lymphoma cells to murine microvascular endothelial cells have been isolated [72]. One of these extracellular calcium-binding proteins has been identified as annexin-II tetramer [71] and antibodies to annexin II tetramer have been shown to antagonize the binding of the RAW117 cells to normal endothelial cells. Many other metastatic cells have also been shown to express annexin II tetramer at concentrations much higher than normal cells, and typically this overexpressed protein is localized to the extracellular face of the plasma membrane. In fact, extracellular annexin II tetramer is absent from most normal cells [70]. The hypothesis has been proposed that extracellular annexin II tetramer expression occurs as a function of cellular transformation and that this protein is involved in divalent cation-dependent adhesion of metastatic cells to their target organ. There is however, reasonable evidence to suggest that annexin-II may also be involved in the adhesion of metastatic cells to their target organ but not in the adhesion process of normal cells [71]. A role for AIIt in cell-cell adhesion has been suggested from an initial study which examined mouse RAW117 large cell lymphoma cell adhesion to mouse liver microvessel endothelial cells. This study demonstrated that mild treatment of the RAW117 cells with 1-butanol resulted lower rates of adhesion to host cell monolayers than for untreated cells [72]. Analysis of the 1-butanol extracts of cell-surface proteins revealed a protein within the range of 37–40 kDa, which was subsequently identified as annexin II tetramer [71]. Extraction of these cells with 1.0 M NaCl did not release AIIt from the plasma membrane of these cells. Furthermore, significant amounts of AIIt were not detected on the surface of the non-metastatic parent cell line. The preincubation of the liver microvessel endothelial cells with exogenous AIIt was reported to block the adhesion of the RAW117 metastatic cells to the substrate cells. Similarly, preincubation of the RAW117 metastatic cells with an antibody to AIIt blocked the adhesion of these cells to the substrate cells. These results suggest that AIIt is expressed on the extracellular surface of mouse RAW 117 liver metastatic cells and this cell-surface AIIt mediates the Ca^{2+}-dependent adhesion of the RAW117 cells to endothelial cells.

One current hypothesis is that the extracellular matrix protein, Tenascin [215] may be involved in the binding of extracellular membrane-bound AIIt to normal cells. However, it unclear how AIIt remains associated with the extracellular face of the plasma membrane of transformed cells. The possibility exists that the binding of AIIt to the outside of the plasma membrane involves the interaction of AIIt with specific membrane receptor(s) (see section 4i). Results based on analysis of many cultured cell lines, suggest that many cancerous cells express elevated levels of annexins which tend to be expressed on the outside of the cell. While a specific role for annexin II has been elucidated in metastatic cell adhesion for some cells, other metastatic cells have been shown to express extracellular annexins other than annexin-II.

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