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Review

Annexins and ATP in membrane traffic: A comparison with membrane fusion machinery°

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Annexins, calcium- and membrane-binding multifunctional proteins, have been implicated in *N*-ethylmaleimide (NEM)-independent fusion of vesicular structures involved in membrane traffic. This view is based on intracellular localization of annexins, which are frequently associated with endosomes, chromaffin granules, caveolae, clathrin-coated pits, and other membrane compartments, engaged in endo- and exocytosis. Moreover, annexins were found to modulate budding and aggregation of vesicle membranes, to interact with cytoskeletal proteins, and, upon binding to membranes, to change the structure of lipid bilayer, leading to membrane fusion. In addition, some annexins are substrates for various protein kinases and, in membrane-bound form, reveal calcium channel activity. Recently, annexins were observed to interact *in vitro* and *in vivo* with nucleotides, ATP, GTP or cAMP, which are potent mediators of membrane traffic processes. In addition, annexin VII showed hydrolytic activity towards GTP, and similarities in the mechanism of action to that of small GTP-binding proteins were found. The aim of the present review is to summarize the observations implying annexins as possible effectors in endo- and exocytosis and to compare them with well known complexes of cytosolic and membrane proteins forming the true membrane fusion machinery within a cell, conserved from yeast to the neurons of humans.

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Abbreviations: NEM, *N*-ethylmaleimide; NSF, *N*-ethylmaleimide-sensitive factor; RabGDI, RabGDP dissociation inhibitor; SNAP, soluble NSF-attachment protein; SNAP-25, synaptosomes-associated protein, unrelated to SNAP; SNARE, SNAP receptor, target-associated (t-SNARE) or vesicles-associated (v-SNARE); VAMP, vesicle-associated membrane protein.

In the past years, a large number of observations became accumulated on the molecular machinery by which vesicle budding, targeting, docking and fusion in secretory pathways of membrane vesicular transport in all eukaryotic cells is accomplished [1]. It is widely accepted that the so-called membrane fusion machinery consists of three major protein complexes: the Rab3A-Rabphilin-3A, the NSF-SNAP-SNARE, and the fusion pore formation complex [2–4]. It has to be stressed that the same molecular machinery is utilized in many vesicular transport pathways, including intra-Golgi transport, transport from the endoplasmic reticulum to the Golgi complex, fusion between endosomal vesicles, and neurotransmitter release at the synapses of the central nervous system, and it is well preserved from yeast to the neurons of humans [1, 5]. This molecular machinery is regulated by various factors, amongst them, ATP, GTP, cAMP and calcium [6, 7]. Each of these factors has its own specific target. In addition, there is a growing number of observations concerning the role in some membrane traffic processes, of annexins, calcium- and phospholipid-binding proteins which have recently been discovered to be nucleotide-binding proteins [8–12].

Vesicular transport has been most extensively studied in the central nervous system of mammals [13]. Specialized cell–cell contact sites (synapses) contain the whole machinery for neurotransmitter release and response, allowing for the reciprocal transmission of information between adjacent cells [5, 13, 14]. A major advance in the analysis of transmitter release mechanisms has been the cloning and characterization of several proteins that may participate at different stages of the release cycle [15]. A mechanism of regulated trafficking has also been recently implicated in the ligand-regulated transport of the Menkes copper P-type ATPase efflux pump from the Golgi apparatus to the plasma membrane [16, 17] and in cell detoxification processes, e.g. active transport of glutathione conjugates of endo- and xenobiotics into the bile against their concentration gradient [18–21].

CYTOSOL AND MEMBRANE FACTORS OF REGULATED VESICLE TRANSPORT

Three major steps in membrane–membrane interactions during vesicular trafficking are observed: vesicle budding, targeting, and fusion [22]. Targeting of transport vesicles is triggered by synapsins. These proteins constitute about 9% of the total protein of the synaptic vesicle membrane. Synapsins (Ia, Ib, IIa and IIb) are encoded by two genes. They differ primarily in the C-terminal end and they all can be phosphorylated near their N-terminal end by cAMP-dependent protein kinase and/or Ca^{2+}/calmodulin kinase I. In addition, synapsins Ia and Ib are phosphorylated by calmodulin kinase II near their C-terminal end; this target site is missing in the shorter synapsins IIa and IIb. The major role of synapsins is in determining the status of synaptic vesicles by interacting with the cytoskeleton, irrespective whether they are in the free pool, available for binding to the presynaptic membranes, or not. Phosphorylation of synapsins prevents the binding of synaptic vesicles to the cytoskeleton or releases vesicles from their binding sites [5, 13]. Recently, it has been shown that synapsins I and II contain an ATP-binding domain, and ATP binding is differentially regulated by Ca^{2+} [23, 24]. Targeting of vesicles requires specific receptors on the incoming vesicle and on the acceptor membrane, which provide a docking site [25].

Vesicular transport processes are inhibited at low concentrations of NEM. This is accompanied by accumulation of uncoated transport vesicles, suggesting that NEM inactivates factor essential for fusion [14, 22], which has been identified as a cytosolic ATP-binding protein, the NEM-sensitive factor (NSF). Readdition of NSF, accomplished by mixing un-

treated Chinese hamster ovary cell extracts with NEM-inhibited cells, reactivated the transport of accumulated vesicles. This strategy was used to purify NSF from those cells. NSF was shown to be a homotrimer formed of subunit of a single type of 76 kDa [26]. Within the cell NSF is found in both a soluble form within the cytosol and as a Golgi apparatus peripheral membrane protein. It is released from membranes upon hydrolysis of ATP, and itself catalyses ATP hydrolysis; the predicted sequence of the NSF protein contains consensus motifs for ATP binding [26].

Membrane binding of NSF *in vitro* is specific and saturable, requiring not only a heat-sensitive integral membrane receptor but also one or more soluble NSF-attachment proteins (SNAP), peripheral membrane components. SNAP was purified from bovine brain tissue in three isoforms (α, β, γ) of 35, 36 and 39 kDa, respectively. Each form is able to partially restore SNAP-dependent transport activity and mediate NSF binding to Golgi membranes [26]. By use of the purified proteins, it was demonstrated that the interaction between NSF and SNAP does not occur in solution but requires SNAP to first interact with membranes. Although each of the SNAP isoforms shares the same general biochemical properties with regard to transport and mediation of NSF binding, they differ in their specific activities and other properties [1].

One of the models of vesicle targeting postulates that the specificity of the process is generated by complexes that form between membrane proteins on the transport vesicle (v-SNARE) and membrane proteins on the target membrane (t-SNARE) where SNARE is a SNAP receptor [1, 14, 25, 27] (Fig. 1A). v- and t-SNARE were originally thought to be localized to vesicle and target membranes, respectively, although a fraction of t-SNARE was also found on vesicles. This suggests that v- and t-SNARE pairing may occur on the same membrane [2]. The formation of the SNARE complex is responsible for the sequential recruitment of NSF and α-SNAP. The ATPase catalytic activity of NSF leads to the disruption of the SNARE complex, and to membrane fusion. The SNARE complex is composed of three synaptic membrane proteins: a v-SNARE protein (VAMP, i.e. vesicle-associated membrane protein, also known as synaptobrevin) and two t-SNARE proteins localized in the presynaptic plasma membrane (syntaxin and SNAP-25, i.e. synaptosome-associated protein, of 25 kDa) [25, 28, 29]. The VAMP family consists of two small proteins of 18 kDa and 17 kDa, anchored to the cytoplasmic side of the synaptic vesicles membrane through a C-terminal domain. These proteins are involved in vesicle transport and in the release of neurotransmitter from presynaptic neuron [5, 13] and in the release of insulin from insulin-containing secretory granules [7].

Syntaxins are integral proteins of presynaptic plasma membranes. They bind synaptotagmin and mediate its interaction with voltage-dependent calcium channels at the site of neurotransmitter release [1]. These channels can directly interact with the G-protein $\beta\gamma$ complex, this resulting in inhibition of the channel [30]. Synaptotagmin is a calcium-sensitive regulatory factor. It is an integral protein of the synaptic vesicle membrane which interacts in a Ca^{2+}-dependent manner with targets localized on the presynaptic membrane (syntaxins 1a and 1b). It is involved in the process of docking of the synaptic vesicle to the presynaptic membrane [1, 2].

FORMATION OF THE FUSION PORE COMPLEX

Exocytosis of a secretory vesicle in neurons and neuroendocrine cells is triggered by an increase in the cytosolic concentration of Ca^{2+}, involves various Ca^{2+}-binding proteins with different affinities for Ca^{2+}, and is followed by endocytic membrane retrieval [31]. The final, most mysterious step in the sequence of events leading to exocytosis is the fusion of a

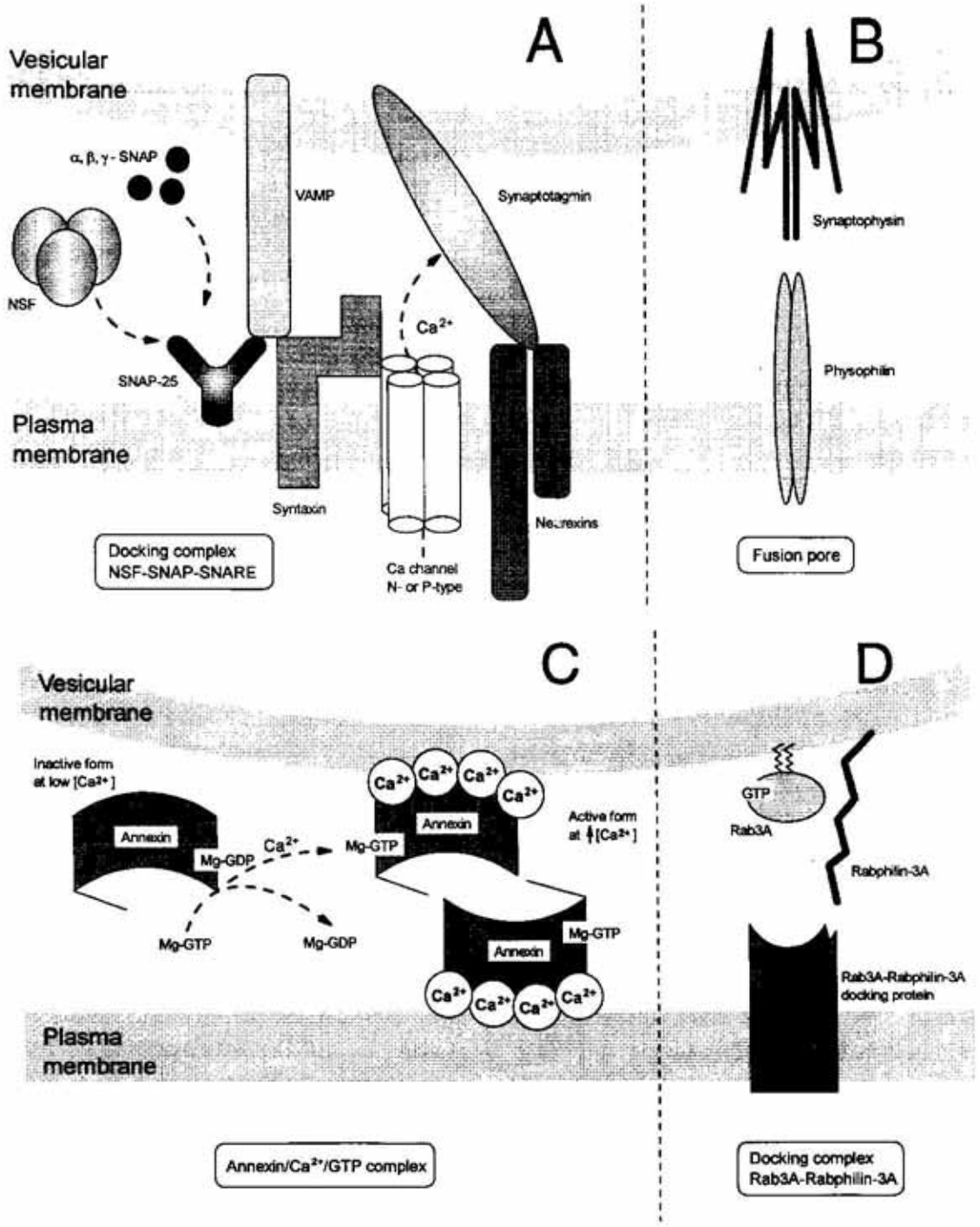

Figure 1. The molecular complexes of proteins postulated to play the role of a hypothetical membrane fusion machinery in the cell.

(A) The NSF-SNAP-SNARE complex; (B) the fusion pore formation complex; (C) it is postulated that nucleotide-binding proteins, annexins, are involved in an NSF-independent fusion event; (D) the Rab3A-Rabphilin-3A complex. Other explanations are in the text.

vesicle with plasma membrane [32]. Fusion can be defined as the set of events that follow docking and include lipid bilayer intermixing of vesicle and target membranes [14]. During fusion, an aqueous connection, termed the fusion pore, forms between the transport vesicle lumen and the space surrounded by the target membrane [33]. Through this pore neurotransmitters or other secretagogues are released to the extracellular millieu. The fusion

pore may be constituted by synaptophysins, integral proteins of synaptic vesicles (Fig. 1B), structurally similar to the gap junction proteins [1, 34]. The process starts with opening of a narrow fusion pore whose conductance and diameter are similar to those of a large ion channel. The molecular structure of the initial fusion pore is not fully solved. It is suggested that it is not built only from lipids, but is surrounded by a protein ring which ultimately expands recruiting lipids into the interior of the fusion pore [33].

Fundamental mechanisms of membrane fusion are conserved over different vesicular pathways in eukaryotic cells. Proximity models postulate that proteins mediate fusion of lipid bilayers by bringing them into close contact, and then bilayers fuse either on their own, or a fusion-catalyzing protein engages the two apposed bilayers using its hydrophobic domains, and pulls them together [32, 33, 35]. In proximity models, fusion is preceded by hemifusion, an intermediate state in which the apposed leaflets from either of the two bilayers partially mix and form a new hybrid bilayer that separates the compartments. This bilayer is later punctured to form an aqueous pore. In another model, called the fusion pore model, the gap between the fusing membranes is bridged by a protein complex entering, or even spanning, both bilayers. Upon activation, the complex made of protein subunits undergoes a conformational change allowing formation of an aqueous channel that connects the two fusing compartments. The subunits move apart radially, exposing amphipathic surfaces, stimulating the migration of lipids and expanding the pore [33].

ANNEXINS – NUCLEOTIDE-BINDING PROTEINS

Annexins, a family of soluble or membrane-associated, calcium-binding proteins encoded in mammals by ten genes (as reviewed in [36, 37]) very likely are also involved in vesicular traffic [38, 39]. This idea arose from the observation that annexin II aggregates *in vitro* liposomes and enhances fusion of secretory granules at micromolar Ca^{2+} concentrations, similar to those found to activate exocytosis and NSF-independent fusion of early endosomes [40]. Annexin II has been shown to be associated with the inner surface of the plasma membrane in adrenal chromaffin cells and fibroblasts. It was also detected on clathrin-coated pits and on early endosomes in Baby hamster kidney cells. In Madin-Darby canine kidney cells and in hepatocytes, annexin II was found to be co-localized with early endosomes and the receptor-recycling compartment, and to determine the endosome spatial distribution [41–44]. Annexin II was proposed to crosslink secretory granules to the plasma membrane in regulated exocytosis, allowing the bilayers to approach each other and fuse [42]. In J774 macrophages, calcium-dependent fusion of early endosomes was reduced by an antiserum against annexin II [42], while binding to phagosomes was prevented by washing them with a calcium chelator [45].

In chromaffin cells and upon nicotine stimulation, annexin II redistributes within different membrane compartments; this is accompanied by phosphorylation of annexin II and catecholamine release from chromaffin granules [46]. Fractionation of cellular proteins with detergent causes annexin II to partition, forming an octylglucoside-soluble fraction which contains integral proteins from the plasma and granule membranes. This fraction may represent a special membrane compartment where SNAP, annexin II and Rab3A become concentrated upon stimulation of the cell. The localization of annexin II in such a fraction of proteins is consistent with its role on the granule and target membranes as a factor required for the close apposition of these two membranes in exo- and endocytosis [46].

Annexin VI was detected in endothelial cell plasma membranes, both with associated caveolae and with membranes stripped of the

caveolae. The presence of annexin VI in the latter membranes is consistent with its bilateral function in diminishing local repulsive forces between the membranes, and in creating a local perturbation of the lipid bilayer that permits the fusion of two distinct membranes [47, 48]. Annexin VI is the largest annexin (68 kDa) [49], and it is abundant in most human tissues studied to date, especially in the secretory epithelia and endocrine cells [49]. Annexin VI is localized within a cell on the surface of plasma membranes. It is also associated with mitochondria [50] and with intracellular organelles involved in sequestering and release of Ca^{2+}, e.g. chromaffin granules [49]. In hepatocytes, annexin VI is also associated with the endosomal fraction containing multivesicular bodies and compartments for uncoupling receptors and ligands [47, 51, 52]. This localization of annexin VI may be of significance for intracellular trafficking of endosomes; it has also been suggested that it is required for the budding of clathrin-coated pits [53].

Taking into account all the above mentioned results, annexins appear to act as mediators of exocytosis since they are found to stimulate vesicle aggregation and fusion in a calcium-dependent manner [54, 55]. This is consistent with the observation that exocytosis is often preceded by a rise in cytosolic Ca^{2+} concentration. In adrenal medulla, annexins are involved in the aggregation of chromaffin granules [56]. Interestingly, the aggregation of chromaffin granules and phosphatidylserine liposomes driven *in vitro* by annexin I, and the ability of the latter to form calcium channels have been found to be modulated by cAMP (which increases its cooperativity and maximal velocity) and ATP (which inhibits the aggregation, without affecting the cooperativity) [8].

Although the binding of annexins to membranes seems to be regulated by calcium [37, 57], various annexin isoforms have been found to bind to the membranes even when intracellular Ca^{2+} concentrations reach low resting levels. In bovine heart, lungs and brain some annexins partitioned in the micellar phase after nonionic detergent treatment [58]. On the other hand, in the presence of Ca^{2+} annexin VI was resistant to treatment with detergents and, in particular, it colocalizes with the actin binding proteins, α-actinin and fimbrin [59]. ATP at a physiological concentration range potentiated association of annexin VI with the hepatocyte plasma membrane, especially at submillimolar concentrations of Ca^{2+} [60]. In addition, the calcium-dependent relocation of annexin V to plasma membrane upon platelet stimulation was enhanced by ATP; although it has been suggested that it is phosphorylation which plays a role [61]. Moreover, the Ca^{2+}-dependent interaction of annexin VI with erythrocyte ghosts and with F-actin is modulated *in vitro* by ATP at physiological concentrations [10]. In addition, ATP has been found to modulate annexin VI-driven aggregation of phosphatidylserine liposomes [62]. This has not been observed in the case of a homologous protein, annexin IV [63]. Finally, biochemical evidence has been provided that annexin VI binds nucleotides, as revealed by photoaffinity labeling of annexin VI with the ATP analog 8-azido-[γ-^{32}P]ATP, and by the increase of the extrinsic fluorescence of 2′-(or 3′)-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate in the presence of the protein [10, 11].

These observations seem to indicate that not only binding of Ca^{2+} to the protein molecule but also other ligands, such as nucleotides, may influence the interaction of annexins with membranes (for review see [64]). One has to remember, however, that annexins in their structure contain neither the A nor the B Walker consensus motifs characteristic of ATP/GTP-binding proteins [65], although they share partial homology with cyclic nucleotide-binding proteins [8]. The strongest arguments supporting the idea of annexins being ATP-binding proteins come from *in vitro* observations that suggest that a nucleotide-binding domain may exist within the annexin

VI molecule. However, annexin VI exhibits structural features different from those found in other ATP-binding proteins [66]. The nucleotide-binding domain of annexin VI is probably localized in a hydrophobic pocket between two symmetric lobes of the protein molecule, each consisting of four Ca^{2+}- and phospholipid-binding domains [66], in analogy to the hydrophobic pockets within the actin molecule and in other related proteins [67]. The connector between these lobes may participate in the creation of an ATP-binding domain, since in most of annexin VI genus-specific isoforms it contains a unique Trp343 residue [66]. These speculations are strengthened by indirect evidence that the binding of ATP to annexin VI implies a rearrangement within the protein molecule's tertiary structure leading to a change in exposure of the Trp343-containing domain [11, 66], and also by the observations made for other ATP-binding proteins revealing the importance of a particular tryptophan residue in the stabilization of the adenine ring of nucleotides while bound to the ATP-binding pocket of the protein [68].

Annexins, upon binding to membranes, were found to form ion-selective voltage-dependent channels conducting in most cases calcium ions [69–73]. This implies that annexins must possess structural and functional features of both soluble and integral membrane proteins [74], since at least partial penetration of soluble annexin into a lipid bilayer is a prerequisite for the channel activity. If so, their interaction with membranes may stimulate fusion processes, as suggested for example in the case of annexin XII hexamer forming in membranes an ion channel, which, unlike the calcium-selective channel formed by annexin V, is probably selective to anions [71]. In addition, the structure of the annexin calcium-binding site implies a preference of annexin for phosphatidylserine [70, 75, 76]. It is, therefore, possible that ATP may mimic the polar head-group of phospholipid upon binding to annexins, and, furthermore, modulates their interaction with membranes. It is worth noting that a key domain in an annexin molecule that stimulate Ca^{2+}-dependent exocytosis has been identified in chromaffin cells [77]. This observation is supported by the discovery, in the annexin II molecule, of a specific protein domain which is reponsible for binding of annexin to membrane and may play a role in specific interaction of annexin II with early endosomes [44].

In addition, annexin VII has been found to be both a Ca^{2+}-dependent GTP-binding protein and a GTPase [9]. Since GTP and its non-hydrolysable analog GTPγS are known to promote Ca^{2+}-dependent exocytosis in many cell types by a mechanism thought to involve as yet unknown proteins, Pollard and his co-workers [9] hypothesized that annexin VII is one of those proteins. They used streptolysin *O*-permeabilized cells and found that the initial rate of annexin VII-driven Ca^{2+}-dependent aggregation of chromaffin granules and phosphatidylserine liposomes was increased by GTPγS > GTP, while other trinucleotides, GDP and GMP were without effect [9]. GTP also influenced liposome fusion driven by annexin VII without changing the apparent $K_{1/2}$ for calcium. Pollard and his colleagues suggested that annexin VII binds GTP, and, as an annexin VII–GTP complex, is active and reveals fusogenic activity. Upon Mg-GTP hydrolysis the protein becomes inactivated, but can be reactivated by elevation of the intracellular Ca^{2+} concentration to above 50 μM [9] (Fig. 1C). It has to be stressed that annexin VII is not the only Ca^{2+}- and GTP-binding protein. In some characteristics, annexin VII seems to resemble the Ca^{2+}- and GTP-binding protein of human fibroblasts, calexcitin [78].

OTHER SMALL GTP-BINDING PROTEINS

The involvement of various types of GTP-binding proteins and GTPases in coordinating vesicular transport and coat assembly of

transport vesicles is well known. It is based on non-selective effects of nonhydrolysable analog of GTP, and AlF_4^-, a phosphate analog of GTP, specifically activating members of the heterotrimeric G protein family: both nonhydrolysable analog of GTP and AlF_4^- inhibit intracellular transport between various compartments within the cell [79]. It is worth noting that in the case of fusion mediated by annexin VII, the process was actually activated by GTPγS, AlF_4^- and mastoparan [9, 12]. A prominent example of an GTP-binding protein involved in vesicular transport is Rab3A in the Rab3A-Rabphilin-3A complex which regulates the targeting and docking of the synaptic vesicles at the active zone of the synapse. Rab3A belongs to a large family of small GTP-binding proteins [2, 80], is specific for synaptic vesicles and is involved in docking and fusion of vesicles with postsynaptic membrane in exocytosis. This protein is anchored to the membrane of synaptic vesicle through a polyprenyl side chain near its C-terminal end and plays the role of a molecular switch in the complex [81]. Rab3A in the GDP-bound form is inactive and can be recognized by the RabGDP dissociation inhibitor (RabGDI) which specifically binds to the GDP-bound form of Rab3A and translocates it from the membrane to the cytoplasm. In the GTP-bound form Rab3A does not interact with RabGDI, the protein becomes active and is recognized by Rabphilin-3A, localized in the cytoplasm. Then, Rab3A in complex with Rabphilin-3A interacts with Rab3A–Rabphilin-3A docking protein at the postsynaptic membrane (Fig. 1D), accomplishing fusion of the synaptic vesicle with the target membrane [2, 80, 81].

CONCLUDING REMARKS

The existence of a large number of membrane traffic processes, some of them highly specialized, e.g. neurotransmitter release, and of many regulating factors, indicate that multiple and/or multifunctional protein complexes are involved. It is now obvious that along with the well-recognized NSF-SNAP-SNARE complexes forming a true membrane fusion machinery, other proteins may catalyse NSF-independent processes in specialized cells. Among many others, annexins seem to be well suited to play such a role. First of all, they are calcium- and phospholipid-binding proteins which relocate within the cell upon stimulation. They are substrates for various protein kinases and they modulate the activity of phospholipases involved in signal transduction. Their calcium-dependent binding to membranes evokes many structural events, for example changes in the permeability of membranes to ions, and enhancement of membrane fusion. Moreover, annexins were found to be attached to endosomes, secretory granules and clathrin-coated pits, i.e. structures engaged in membrane traffic. Recently, some annexins were found to bind either ATP (annexins VI and IV) or GTP (annexin VII) or cAMP (annexin I), and, for annexin VII, a hydrolytic activity towards GTP has been observed. Such an activity is thought to play a role of a molecular trigger switching "on" and "off" the annexin function within a cell. What remains to be elucidated is the functional meaning of binding of nucleotides to annexins and the interrelationship between annexins and other members of the membrane fusion machinery. The finding that annexins may colocalize at the membrane compartment involved in exocytosis with SNAP and Rab3A supports the latter possibility.

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