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THE CYTOSKELETON AS A BARRIER TO EXOCYTOSIS IN SECRETORY CELLS

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Summary

Chromaffin cells of the adrenal medulla synthesize, store and secrete catecholamines. These cells contain numerous electron-dense secretory granules which discharge their contents into the extracellular space by exocytosis. The subplasmalemmal area of the chromaffin cell is characterized by the presence of a highly organized cytoskeletal network. F-Actin seems to be exclusively localized in this area and together with specific actin-binding proteins forms a dense viscoelastic gel; fodrin, vinculin and caldesmon, three actin cross-linking proteins, and gelsolin, an actin-severing protein, are found in this subplasmalemmal region. Since fodrin-, caldesmon- and alpha-actinin-binding sites exist on secretory granule membranes, actin filaments can also link secretory granules. Chromaffin granules can be entrapped in this subplasmalemmal lattice and thus the cytoskeleton acts as a barrier preventing exocytosis. When cells are stimulated, molecular rearrangements of the subplasmalemmal cytoskeleton take place: F-actin depolymerizes and fodrin reorganizes into patches. In addition, introduction of monospecific antifodrin immunoglobulins into digitonin-permeabilized cells blocks exocytosis, demonstrating the crucial role of this actin-binding protein. In bacterial toxin-permeabilized chromaffin cells, experiments using actin-perturbing agents such as cytochalasin D and DNAase I suggest that exocytosis is in part controlled by the cytoskeleton. The intracellular signal governing the cytoskeletal reorganization (associated with exocytosis) is calcium. Calcium inhibits some and activates other actin-binding proteins and consequently causes dissolution of the subplasmalemmal cytoskeleton. This dissolution of cytoskeletal filaments should result in granule detachment and permit granules free access to exocytotic sites on the plasma membrane.

Introduction

Chromaffin cells, which play an important role in stress situations, have been a favourite subject for the study of secretory mechanisms. These cells, which are localized in the medullary part of adrenal glands, secrete catecholamines together with polypeptides and neuropeptides when the acetylcholine-specific receptor is stimulated. Catecholamines are packaged into secretory granules and released from the cell by exocytosis: a mechanism which implies the fusion of the granule

Key words: exocytosis, cytoskeleton, chromaffin cells.

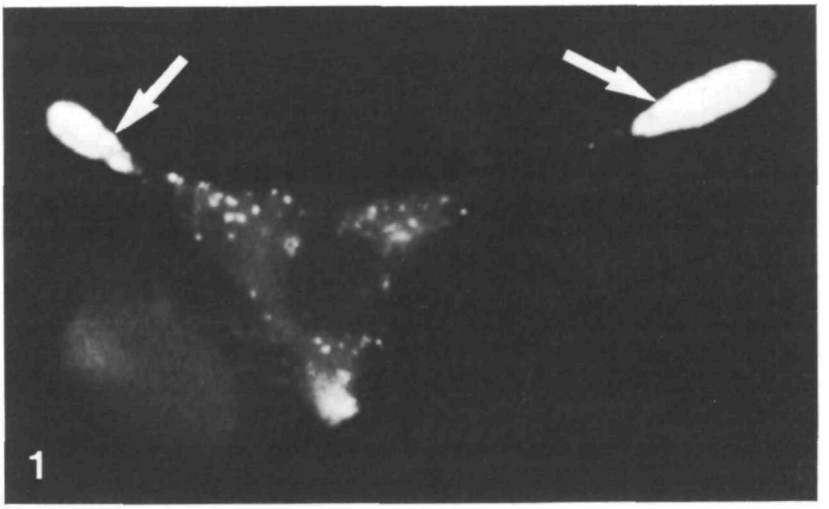

Fig. 1. Immunocytochemical localization of secretory granules in chromaffin cells in culture. Bovine adrenal chromaffin cells maintained in cultures for 7 days were labelled with an antibody directed against protein marker of secretory granules, chromogranin A (Ehrhart *et al.* 1986). Note the accumulation of granules in the expansion tips (arrowheads). Magnification, ×1100.

membrane with the cell plasma membrane and the transient formation of a membrane continuum.

Two types of secretion have recently been described: regulated and constitutive (Kelly, 1985). Release of stored material in each type of secretion occurs by exocytosis. Regulated secretion requires specific stimuli and a temporary increase in the intracellular concentration of free calcium. However, the fusion of a constitutive secretory vesicle with the plasma membrane requires neither extracellular signals nor elevation of calcium concentration. Therefore, regulated secretion must be controlled at a stage prior to exocytosis. Recent experiments that we and others have carried out on bovine chromaffin cells demonstrate that such control is probably exerted by the cytoskeleton in the subplasmalemmal space. In this strategic zone of the cell, the cytoskeleton acts as a barrier, preventing membrane interactions in resting cells.

The cytoskeleton in endocrine cells

Chromaffin cells release catecholamines at their basolateral surface. This implies that the secretory granule has to be directed to this zone of exocytosis. Cell polarity in chromaffin cells is exemplified when cells are maintained in primary culture in conditions that favour the formation of long cell processes (Aunis, Guérold, Bader & Ciesielski-Treska, 1980; Hesketh, Ciesielski-Treska & Aunis, 1981). In these cells chromaffin granules were found to accumulate in neuri[illegible] endings with discrete localization in varicosities along the trunk (Fig. 1). A similar

finding has also been reported in cultured ATt-20, an ACTH-secreting cell line (Kelly, 1985).

The observation that secretory granules are found in certain cell areas and accumulate in expansion tips indicates that the granules are transported and locally sequestered; supramolecular structures must then exist which entrap the granules, once they are located in preferential cell areas, and prevent them from moving back by Brownian movement.

Using conventional electron microscope techniques, a prominent cytoskeleton can be revealed in epoxy-embedded preparations of adrenal medulla (Poisner & Cooke, 1975). In cultured chromaffin cells, microtubules are abundant in the Golgi domain, in neurite-like expansions and in the subplasmalemmal space (Bader *et al.* 1981; Aunis, Bader, Langley & Perrin, 1987*a*). After embedding in hydrophilic resins, cytoskeletal structures are clearly visible forming a three-dimensional network composed of filaments which interconnect membrane-limited organelles, microtubules and cell membrane. This network appears more dense in the subplasmalemmal space (Aunis *et al.* 1987*a*).

Using the freeze-etching technique, after classical fixation and glycerol exchange (Aunis, Hesketh & Devilliers, 1979), connections between secretory granules and the plasma membrane were described in chromaffin cells. These connections are proteinaceous and probably of a filamentous nature, since in pancreatic β-cells, peripheral microfilamentous structures have been reported to cross-link granule and plasma membranes (Orci, Perrelet & Friend, 1977). The three-dimensional architecture of the subplasmalemmal space of secretory cells was recently revealed by the elegant quick-freeze, deep-etch technique. Fine cross-bridges were observed between adjoining microtubules, membrane-limited organelles, filaments and the cell membrane in permeabilized platelets (Nakata & Hirokawa, 1987). Some of the filaments were shown to be actin filaments since they could be decorated with subfragment 1 of myosin. Interestingly, actin filaments were linked with the plasma membrane *via* short filaments and occasionally ended on the cell membrane.

The main conclusion from these morphological studies is that the subplasmalemmal zone of secretory cells is composed of a complex, three-dimensional network to which organelles and secretory granules appear to be linked.

Organization of the subplasmalemmal space

Ultrastructural analysis indicates that the subplasmalemmal space is enriched with cytoskeleton elements, an observation which was confirmed using immunocytochemical techniques with specific antibodies. For example, fodrin localization in chromaffin cells has been investigated using a specific immunoaffinity-purified antibody directed against the α-chain of fodrin (Perrin & Aunis, 1985).

Fodrin, which is a member of the spectrin family of proteins (Goodman & Shiffer, 1983) found in non-erythroid cells (Glenney, Glenney & Weber, 1982*b*), is a calcium-regulated protein which binds to and cross-links F-actin (Glenney,

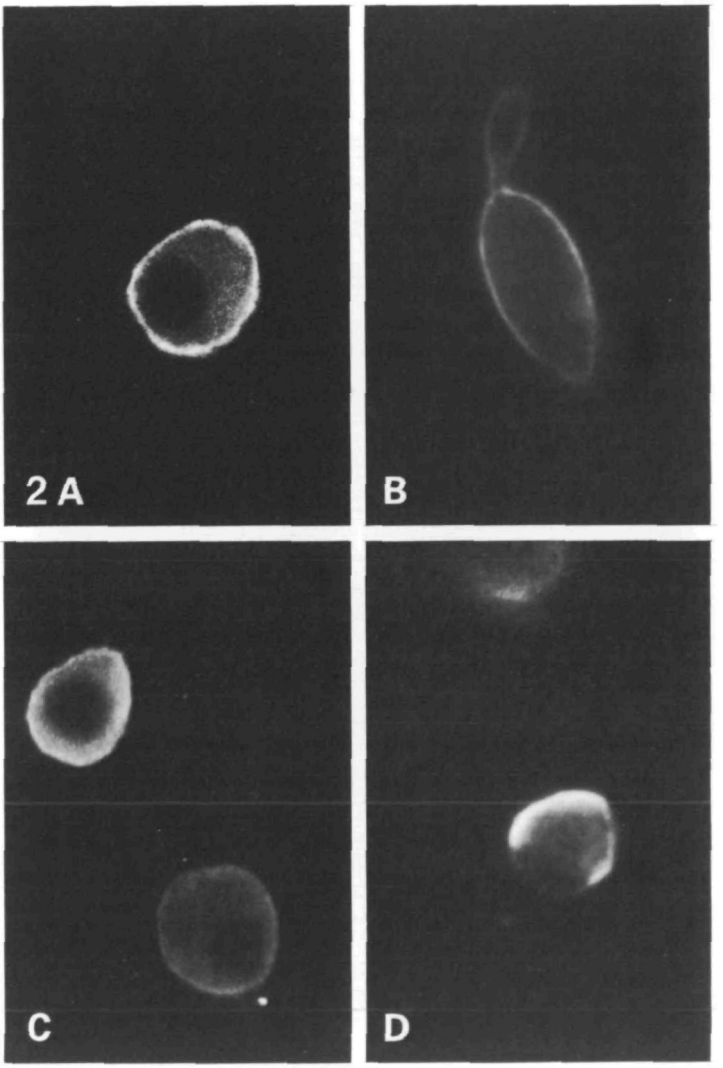

Fig. 2. Immunocytochemical localization of fodrin (A), vinculin (C) and gelsolin (D) and cytochemical localization of F-actin with rhodamine-coupled phalloidin (B). All antibodies have been purified by immunoaffinity on nitrocellulose strips (Perrin & Aunis, 1985) and were used on cultured bovine chromaffin cells. Rhodamine-coupled phalloidin, a specific ligand of F-actin, and anti-gelsolin antibody were used on streptolysin-O-permeabilized chromaffin cells (Sontag, Aunis & Bader, 1988). Magnification, ×820.

Glenney, Osborn & Weber, 1982*a*). In bovine chromaffin cells maintained in primary culture, fodrin was found to be exclusively localized in the subplasmalemmal space: an intense fluorescent ring was visible at the cell periphery when cells were labelled with anti-α-fodrin immunoglobulins (Fig. 2; Perrin & Aunis, 1985).

This localization was then confirmed at the ultrastructural level with the indirect peroxidase method on vibratome slices of the adrenal gland from the rat. The electron-dense precipitate indicative of fodrin was clearly limited to a narrow zone adjacent to the plasma membrane (Langley, Perrin & Aunis, 1986). Though this zone was found to be variable in depth, it extended, on average, for about 230 nm from the membrane into the cytoplasm. This is approximately the length of the fodrin molecule, which appears by rotary shadowing as a long, flexible rod 200 nm long (Glenney, Glenney & Weber, 1982*c*).

Many immunocytochemical studies have been performed to look for the localization of actin in secretory cells (Aunis *et al.* 1980; Lee & Trifaro, 1981). However, relatively poor anti-actin antibodies raised against actin purified from muscles have been employed. Moreover, cells are often fixed and treated using very drastic conditions, so that their cytoskeleton and actin filament organization were probably perturbed. It is only recently, using rhodamine-coupled phalloidin (which binds specifically to F-actin and not to G-actin) that the localization and organization of actin has been determined with greater precision. Using bovine chromaffin cells (maintained in primary culture and permeabilized with the streptolysin-O-exotoxin, in mild experimental conditions that preserve intracellular structures), we were able to demonstrate that rhodamine-coupled phalloidin is found exclusively at the cell periphery, forming a typical fluorescent ring (Fig. 2; Sontag, Aunis & Bader, 1988). This preferential localization of F-actin in the subplasmalemmal space has also been shown in intact cultured chromaffin cells (Cheek & Burgoyne, 1986). Thus, actin filaments are found beneath the cell membrane, a localization which parallels that of fodrin, one of the actin-binding proteins. This observation raises the question of whether any other actin-binding proteins are preferentially localized in this cell zone.

As yet few cytoskeletal proteins have been characterized in secretory cells. Vinculin, an actin-binding protein which is thought to link actin filaments to plasma membrane (Geiger, 1983), has been immunocytochemically characterized in the subplasmalemmal space of chromaffin cells (Fig. 2). Caldesmon, a calmodulin-regulated protein (Sobue, Muramoto, Fujita & Kakiuchi, 1981) known to bind reversibly to F-actin, is also exclusively localized at the periphery of chromaffin cells in culture (Burgoyne, Cheek & Norman, 1986). Finally, gelsolin, originally described in macrophages as an actin-binding protein that severs actin filaments depending on calcium concentration (Yin & Stossel, 1979), has also been localized at the cell periphery in streptolysin-O-permeabilized chromaffin cells (Fig. 2).

However, other actin-interacting proteins have been found in different areas. For example, α-actinin, which cross-links actin filaments (Jockusch & Isenberg, 1981), is distributed throughout the chromaffin cell cytoplasm (Aunis *et al.* 1980).

From all these observations, the subplasmalemmal space appears to be a highly organized cell zone containing most of the cell's filamentous F-actin; the high density of actin filaments together with numerous actin-binding proteins result in the formation of a complex three-dimensional network which must behave as a viscoelastic gel. Since this cell region is highly strategic with regard to exocytosis,

one needs to know whether interactions exist between secretory granules and the highly organized cytoskeleton.

Interaction of secretory granules with the cytoskeleton

Several reviews have recently appeared on this topic (Burgoyne, 1984; Aunis, Perrin & Langley, 1987*b*; Aunis *et al.* 1987*a*; Walker & Agoston, 1987), so here we will summarize the major findings. To date the best model systems for investigating the interaction of the cytoskeleton with the granules have been the *Torpedo* electric organ and more particularly the chromaffin cell. In both systems one can obtain a highly purified preparation of secretory vesicles.

Synaptic vesicles and chromaffin granules have been reported to contain tubulin (Zisapel, Levi & Gozes, 1980; Burke & DeLorenzo, 1982) and to possess tubulin-binding sites (Bernier-Valentin, Aunis & Rousset, 1983). Although the presence of tubulin on storage organelle-limiting membranes has been questioned (Bader *et al.* 1981; Walker & Agoston, 1987), the direct or indirect association of the granules and the synaptic vesicles with microtubules is an important property of their membranes which probably plays a specific role in vesicle translocation from the Golgi domain to the cell periphery or nerve terminals (Sheetz *et al.* 1987). However, no evidence has yet been given for any direct role of microtubules during exocytosis.

Purified cholinergic synaptic vesicles from the *Torpedo* electric organ contain actin as a major component (Tashiro & Stadler, 1978). Synapsin I was also found to be associated with brain synaptic vesicles on their cytoplasmic surface (DeCamilli, Harris, Huttner & Greengard, 1983; Huttner, Schieber, Greengard & DeCamilli, 1983). Synapsin I, which is the most characterized synaptic vesicle-associated protein (DeCamilli & Greengard, 1986), is a phosphoprotein that binds to synaptic vesicle membranes depending on its phosphorylation state. In addition, it is able to interact with spectrin (Baines & Bennett, 1985), with neurofilaments (Goldenring *et al.* 1986) and with microtubules (Baines & Bennett, 1986). Immunological and structural homology of synapsin I with protein 4.1, a component of the erythrocyte membrane cross-linking glycophorin to spectrin and actin (Marchesi, 1985), has been reported (Baines & Bennett, 1985). All these recent studies and others (see below) argue for a role of synapsin I in synaptic vesicle interaction with the cytoskeleton present in nerve terminals (LeBeux & Willemot, 1975). However, these considerations cannot be extended to secretory cells since immunocytochemical studies show synapsin I to be absent from chromaffin cells (DeCamilli *et al.* 1983).

Isolated chromaffin granule membranes contain actin (Burridge & Phillips, 1976; Meyer & Burger, 1979) and actin-binding sites are probably present on granule membranes. α-Actinin has been shown to be present in chromaffin granule membranes (Jockusch *et al.* 1977; Aunis *et al.* 1980). This actin-binding protein stabilizes actin nuclei in membranes and promotes the assembly of F-actin from G-actin (Bader & Aunis, 1983). Isolated granule membranes have been shown to

interact with actin filaments (Burridge & Phillips, 1976; Wilkins & Lin, 1981; Fowler & Pollard, 1982; Aunis & Perrin, 1984) and this calcium-dependent interaction is probably due to the fodrin which is present in purified chromaffin granules (Aunis & Perrin, 1984).

Fodrin is able to bind to chromaffin granule membranes ($K_d = 1{\cdot}4 \times 10^{-8}$ mol l^{-1}; Aunis *et al.* 1987*a*) and there is a good correlation between the increase in the viscosity of F-actin solutions and the binding of fodrin to granule membranes. We have looked for the protein responsible for fodrin binding on chromaffin granule membranes (D. Perrin & D. Aunis, unpublished results). A component with a molecular mass of 35–40 kDa was detectable on polyacrylamide gels using [^{125}I]fodrin. Although we suspected p36, a lipid-binding, F-actin-binding and spectrin-binding protein (Gerke & Weber, 1984), also known as lipocortin or calpactin (Glenney, 1986), further identification of the fodrin-binding component has not yet been pursued.

Recently, caldesmon which is a 70-kDa calmodulin-regulated actin-binding protein has been shown to be one member of a group of proteins that bind to the chromaffin granule membrane in a calcium-dependent manner (Burgoyne *et al.* 1986). Therefore this protein could also be responsible for the interaction of the granules with actin, although biochemical studies on the nature of the interaction of caldesmon with actin remain to be done in secretory cells.

In conclusion, the membrane of storage organelles has the capacity to bind to some actin-binding proteins which could be responsible for the binding of chromaffin granules to the cytoskeletal elements. As most of the actin filaments are present in the subplasmalemmal space, together with the majority of the actin-binding proteins, the interaction of the granule membrane with the cytoskeleton in this cell area limits granule diffusion and, therefore, results in an inhibition of exocytosis. Such a structural model implies that the storage granule must be freed from the cytoskeleton to gain access to exocytotic sites. We will discuss next the molecular mechanisms which are activated at the step preceding exocytosis.

Regulation of exocytosis by the cytoskeleton

Two recent reports have shown that the subplasmalemmal cytoskeleton is not a static structure, because molecular rearrangements occur when secretory cells are stimulated. Using immunocytochemical techniques with immunoaffinity-purified antibodies, we were able to show that stimulation of chromaffin cells results in the redistribution of fodrin into subplasmalemmal patches (Perrin & Aunis, 1985). Later, using staining with rhodamine-coupled phalloidin, it was found that actin filaments present in the cortical area of chromaffin cells disassembled very rapidly after stimulation (Cheek & Burgoyne, 1986).

To evaluate further the role of fodrin in the secretory process, we introduced monovalent, immunoaffinity-purified antibody into digitonin-permeabilized chromaffin cells; the fixation of immunoglobulins on their antigenic sites resulted in a partial inhibition (50%) of calcium-induced secretion (Perrin *et al.* 1987).

Although these experiments did not reveal the exact role of fodrin (because polyclonal antibody can bind to many sites on fodrin molecules and thus affect very different functions), they do, however, give support to its role in secretion. Interestingly, some granules did not seem to be affected by the inhibition of fodrin by its antibody, suggesting the involvement of fodrin at a step prior to exocytosis.

Curiously, drugs which bind to actin have no effect on calcium-dependent catecholamine release in either electrically (Baker & Knight, 1984) or digitonin-permeabilized (Lelkes, Friedman, Rosenheck & Oplatka, 1986) chromaffin cells. This might be explained by the fact that prolonged incubations, after permeabilization with digitonin, result in the progressive inhibition of secretion due to the leakage of soluble proteins that are essential for exocytosis (Sarafian, Aunis & Bader, 1987). A similar situation is likely to occur in electrically permeabilized cells. Although the leaked proteins have not yet been identified, with the exception of calmodulin and protein kinase C (Sarafian *et al.* 1987), some of them have been characterized as components of the cytoskeleton (tubulin, actin) and as cytoskeleton-regulating proteins (D. Aunis, D. Thiersé, T. Sarafian & M. D. Bader, unpublished results). Interestingly, fodrin did not leak out of digitonin-permeabilized cells, an observation which correlates well with the fact that anti-fodrin immunoglobulins were able to decrease catecholamine release from digitonin-treated cells (Perrin *et al.* 1987).

To prevent the rapid leakage of proteins through permeabilization holes, a milder treatment was applied. We used two bacterial exotoxins to permeabilize chromaffin cells: *Staphylococcus aureus* α-toxin (Bader *et al.* 1986*a*) and, more recently, *Streptococcus pyrogenes* streptolysin-O (Sontag *et al.* 1988). α-Toxin creates small pores with a diameter of 2–3 nm and streptolysin-O induces the formation of larger pores, without affecting intracellular structures since the cells still responded to calcium after a long incubation time. After permeabilization, we examined the effect of actin-destabilizing agents on secretion from α-toxin- and from streptolysin-O-permeabilized cells (Sontag *et al.* 1988). We found that incubation of streptolysin-O-permeabilized cells with cytochalasin D or DNAase I, two actin filament disrupting agents, provoked respectively 50 % and 30 % activation of calcium-evoked catecholamine release and this activation was effective at low calcium concentrations ($1–10\,\mu mol\,l^{-1}$). Similar results were obtained with cytochalasin D on α-toxin-permeabilized cells. In separate experiments, we have also observed that α-toxin-permeabilized cells are able to secrete more than 50 % of their stored catecholamines at a continuous rate for 1 h without significant alteration of the secretory machinery (Grant, Aunis & Bader, 1987).

All these data – obtained on cells which were permeabilized in conditions that preserved their internal structures, particularly the cytoskeletal organization, and prevented leakage of proteins essential for exocytosis – can be interpreted as the consequence of two existing pools of secretory granules in secretory cells: (i) one of immediately releasable granules which are presumably not under the control of the cytoskeleton (but which could be positioned at the cell membrane by docking of cytoskeletal elements), and (ii) a second pool of granules which are linked to

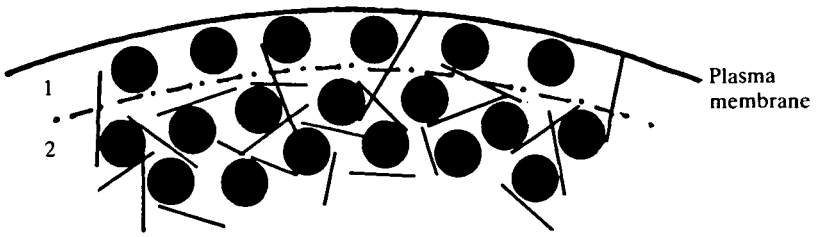

Fig. 3. Subplasmalemmal localization of granule pools in chromaffin cells. One pool of granules (1) is close to the plasma membrane and ready to release stored material; this is the pool which is unaffected by anti-α-fodrin antibody (Perrin, Langley & Aunis, 1987). The second pool (2) is entrapped in the subplasmalemmal cytoskeleton and serves as a reservoir to supply exocytotic demand. The mobilization of this pool is affected by anti-α-fodrin antibody and by actin-destabilizing agents such as cytochalasin D and DNAase I (Sontag, Aunis & Bader, 1988).

cytoskeletal structures by actin filaments, are situated some distance from the plasma membrane and which can be mobilized for a second wave of secretion when the first pool has been exhausted (Fig. 3). Since toxin-treated cells are able

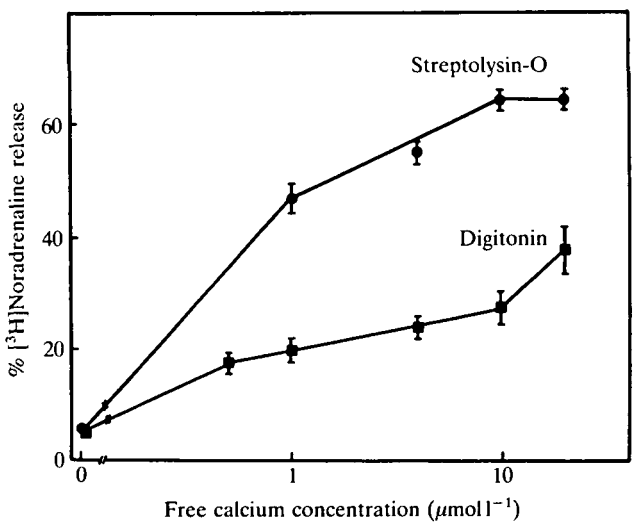

Fig. 4. Calcium-evoked release of catecholamine from streptolysin-O- and digitonin-permeabilized chromaffin cells. Chromaffin cells maintained in primary culture were exposed to either 18 units ml^{-1} of streptolysin-O or 10 μmol l^{-1} of digitonin in potassium glutamate buffer containing 5 mmol l^{-1} Mg^{2+}-ATP at the indicated free calcium concentration. After 10 min of incubation, the medium was removed and [^{3}H]noradrenaline assayed in the medium and remaining cells. Calcium triggered release from both streptolysin-O- and digitonin-permeabilized cells but toxin-treated cells released more catecholamines, indicating that in these cells a larger population of granules is available for release.

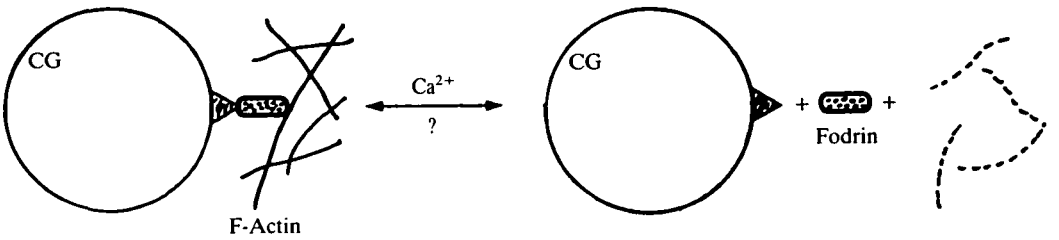

Fig. 5. Model of the interaction of a chromaffin granule (CG) with the cytoskeleton in the subplasmalemmal space. Fodrin cross-links the granule membrane on which a specific receptor might be present with the actin filaments. On stimulation, free calcium concentration increases, thus inhibiting interaction of granule membranes with actin filaments (*via* fodrin detachment ?) and activating gelsolin which provokes shortening of actin filaments. Besides severing the actin–fodrin link, calcium could also activate calmodulin and thus the caldesmon–calmodulin interaction, resulting in destabilization of the actin network.

to release more noradrenaline than detergent-treated cells (Fig. 4), it is likely that the second pool is releasable only when the integrity of the cytoskeletal structure is preserved.

Cheek & Burgoyne (1986) reported that stimulation of intact chromaffin cells induces catecholamine secretion together with disassembly of subplasmalemmal F-actin. Although their quantitative biochemical estimation of the F-actin/G-actin ratio could be erroneous, because of contaminating endothelial actin pools, it nevertheless shows a tendency for F-actin levels to be decreased when cells are depolarized with high K^+ levels or nicotine (Cheek & Burgoyne, 1987). In more recent experiments, we have examined the effect of stimulation on subplasmalemmal F-actin in streptolysin-O-permeabilized chromaffin cells (Sontag *et al.* 1988). Secretion was triggered by micromolar concentrations of free calcium, and we observed that although stress fibres were not altered in contaminating endothelial cells, F-actin disassembled in permeabilized chromaffin cells. These experiments clearly show that calcium is the intracellular signal acting directly or indirectly on actin filaments. This conclusion correlates well with the fact that several calcium-regulated, actin-binding proteins (e.g. fodrin, gelsolin, caldesmon) are present in the subplasmalemmal region together with filamentous actin. We thus propose the following mechanism. When cells are stimulated, intracellular calcium concentration rises (Kao & Schneider, 1986; Cobbold, Cheek, Cuthbertson & Burgoyne, 1987), due to calcium fluxes through voltage-sensitive calcium channels (Artalejo, Garcia & Aunis, 1987), and this transient calcium increase activates F-actin-severing proteins, such as gelsolin (Bader *et al.* 1986*b*), and inhibits F-actin-cross-linking proteins, such as fodrin and caldesmon. This calcium activation of proteins leads to a dissolution of the cytoskeletal barrier with a concomitant release of trapped granules from the cytoskeletal network. Free granules then have access to the plasma membrane (Fig. 5).

Conclusions

Synapsin I has been found exclusively in nerve terminals where it binds to synaptic vesicles. Phosphorylation of synapsin I reduces its affinity for and its detachment from vesicle membranes (DeCamilli & Greengard, 1986). Recent studies argue strongly for a role of synapsin I in the interaction of synaptic vesicle membranes with the cytoskeleton (see above). Homology in the sequence of synapsin I with that of two actin-binding proteins, villin and profilin (McCaffrey & De Genarro, 1986), also favours this view. In addition, interaction of synapsin I with actin is dependent on the state of phosphorylation (Bahler & Greengard, 1987). The role of synapsin I during exocytosis has been demonstrated recently: microinjection of dephospho-synapsin I into the terminal of the squid giant axon decreased the amplitude and rate of rise of the postsynaptic potential, but microinjection of calcium/calmodulin protein kinase II increased release of transmitter (Llinas *et al.* 1985). Therefore in the resting nerve terminal, synaptic vesicles are trapped in the cytoskeletal network by synapsin I bound to vesicle membranes, actin filaments and possibly other structures. Upon depolarization, calcium-calmodulin-dependent phosphorylation of synapsin I results in its detachment from the synaptic vesicles and the consequent detachment of the vesicles from cytoskeletal elements. Dissolution of the cytoskeleton barrier takes place, giving access of the vesicles to exocytosis sites.

The comparison of the situation in the nerve terminal with that in endocrine cells is interesting because it highlights both differences and analogies. Since synapsin I is not present in endocrine cells, the role of linking the granules to the cytoskeletal lattice is probably taken by fodrin, which is able to bind to granule membranes and to actin filaments. We do not know whether fodrin is phosphorylated when the cell is activated. It has been proposed that fodrin–granule-membrane interaction is inhibited by proteases (Burgoyne, 1987). However, recent experiments in our laboratory have failed to show fodrin degradation when chromaffin cells in culture are stimulated with nicotine.

These and future studies at the molecular and cellular level on the interactions between vesicles and granule membranes with cytoskeletal proteins are now being carried out to substantiate our understanding of the mechanisms controlling the inhibitory steps prior to exocytosis and the activating steps leading to the apposition and formation of proteinaceous pores prior to fusion (Breckenbridge & Almers, 1987; Zimmerberg, Curran, Cohen & Brodwick, 1987).

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