Conformational Change and Localization of Calpactin I Complex Involved in Exocytosis as Revealed by Quick-Freeze, Deep-Etch Electron Microscopy and Immunocytochemistry

Takao Nakata,* Kenji Sobue,‡ and Nobutaka Hirokawa*

*Department of Anatomy and Cell Biology, School of Medicine, University of Tokyo, 7-3-1, Hongo, Tokyo, Japan, 113; and ‡Biomedical Education Center, School of Medicine, University of Osaka, Nakanoshima, Osaka, Japan

Abstract. Calpactin I complex, a calcium-dependent phospholipid-binding protein, promotes aggregation of chromaffin vesicles at physiological micromolar calcium ion levels. Calpactin I complex was found to be a globular molecule with a diameter of 10.7 ± 1.7 (SD) nm on mica. When liposomes were aggregated by calpactin, quick-freeze, deep-etching revealed fine thin strands (6.5 ± 1.9 [SD] nm long) cross-linking opposing membranes in addition to the globules on the surface of liposomes. Similar fine strands were also observed between aggregated chromaffin vesicles when they were mixed with calpactin in the presence of Ca^{2+} ion. In cultured chromaffin cells, similar cross-linking short strands (6–10 nm) were found between chromaffin vesicles and the plasma membrane after stimulation with acetylcholine. Plasma membranes also revealed numerous globular structures ~10 nm in diameter on their cytoplasmic surface. Immunoelectron microscopy on frozen ultrathin sections showed that calpactin I was closely associated with the inner face of the plasma membranes and was especially conspicuous between plasma membranes and adjacent vesicles in chromaffin cells. These in vivo and in vitro data strongly suggest that calpactin I complex changes its conformation to cross-link vesicles and the plasma membrane after stimulation of cultured chromaffin cells.

THE exocytotic fusion of membrane vesicles with the plasma membrane is one of the key steps in the process of secretion and transmitter release. In the case of neurons and neuroendocrine cells, this process is triggered upon cell activation by the influx of Ca^{2+} ions into the cells and may involve a reorganization of vesicles and cytoskeleton interactions (Perrin et al., 1987; Hirokawa et al., 1989). However, the molecular targets of Ca^{2+} and detailed mechanisms are still unknown. Adrenal chromaffin cells, which secrete catecholamines on stimulation with acetylcholine, have served as a good model system for the study of exocytosis (Baker and Knight, 1978).

Previous studies using freeze-fracture on exocytotic events have been performed including chromaffin cells (Smith et al., 1973; Aunis et al., 1979). However, these studies all incorporate chemical fixation before freezing, which may change the structure of biological membranes (Chandler and Heuser, 1980). The quick-freezing technique, which enables us to fix living cells within 2 ms without artifacts of chemical fixation, has since been developed for the analysis of these exocytotic events. This technique was the first to reveal clearly that neurotransmitters are released by exocytosis (Heuser et al., 1979). Quick-freezing was applied to other cells, such as mast cells (Chandler and Heuser, 1980) and *Limulus* amebocytes (Ornberg and Reese, 1981). Membrane events in adrenal chromaffin cells during exocytosis were also studied by quick-freezing (Schmidt et al., 1983). However, most of these studies focused on membrane events and involvement of membrane integrated proteins. In the present study, we focused on the cytoplasmic structures involved in exocytosis.

Calpactin I, a major cellular tyrosine kinase substrate (Erikson and Erikson, 1980), which exists in the adrenal medulla (Creutz et al., 1987), is a calcium-dependent phospholipid-binding protein (Gerke and Weber, 1985; Glenney, 1986*a*). This molecule makes the complex $(p36)_2(p10)_2$ with 10K light chains in the cell and aggregates phospholipid-containing liposomes in a calcium-dependent manner (Glenney, 1986*a*,*b*; Glenney et al., 1986, 1987). It also inhibits the activity of phospholipase A_2 (Davidson et al., 1987) and binds actin and nonerythroid spectrin in vitro (Gerke and Weber, 1984). Its physiological role in cells is not yet well understood (Crompton et al., 1988), but a recent study revealed that the calpactin I complex could aggregate chromaffin vesicles with physiological elevation of the calcium concentration to micromolar levels among the other members of the lipocortin family proteins in vitro (Durst and Creutz, 1988). Therefore, this protein appears to be a good candidate for functioning as a cross-linking protein between vesicles and the plasma membrane before exocytotic fusion

of the two membranes (Burgyone, 1988). However, in vivo structural evidence was still lacking, except for some immunofluorescence studies (Greenberg and Edelman, 1983; Burgoyne and Cheek, 1987).

To study the molecular mechanism of exocytosis, we focused on the involvement of calpactin. We first studied in vivo structures of cultured chromaffin cells by quick-freeze, deep-etch EM at the time when exocytosis was induced. With this technique, which allows us to observe the true surface of the membrane (Heuser and Salpeter, 1979; Hirokawa and Heuser, 1981, 1982), we were able to observe cross-linking structures between vesicles and plasma membranes in chromaffin cells.

Secondly, we purified the calpactin I complex molecule and observed its molecular structure, its mode of associations with liposomes, and how it causes the aggregation of liposomes at elevated Ca^{2+} concentration by the quick-freeze, deep-etch rotary replication on fragmented mica. Furthermore, we observed the aggregation of isolated chromaffin vesicles induced by the calpactin I complex. Interestingly, in both reconstitution experiments, we found similar fine strands connecting the two opposing membranes, as observed in chromaffin cells. These structures were apparently different from the molecular structure of the calpactin I complex alone on mica.

Third, we performed immunoelectron microscopy of calpactin I on cryo ultrathin sections of unstimulated and acetylcholine-stimulated chromaffin cells to answer the question: does calpactin I exist between the plasma membrane and the facing surface of the vesicle membrane after activation, as shown in quick-freezing? We discussed the possible involvement of calpactin I in exocytosis based on these data in vivo and in vitro.

Materials and Methods

Isolation and Culture of Chromaffin Cells

Chromaffin cells were isolated from the bovine adrenal medulla with a slight modification of the procedure of Waymire et al. (1983) (Kumakura et al., 1986). Briefly, isolated medulla were perfused retrogradely with Hanks' balanced solution (Gibco Laboratories, Grand Island, NY) containing 0.025% collagenase (Wako Pure Chemical Industries, Osaka, Japan) and 1.33 mg/100 ml DNase I (Sigma Chemical Co., St. Louis, MO) for 1 h at 35°C under 5% CO_2–95% O_2 gas bubbling. The suspension was filtered through a nylon mesh and then centrifuged at 200 *g* for 8 min at room temperature. The sediment of cells was resuspended in 1% BSA Hanks' buffer. This suspension was centrifuged at 120 *g* for 8 min and the pellet was resuspended to $\sim 5 \times 10^5$ cells/ml in DME medium (Gibco Laboratories) containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 40 mg/l gentamycin, 100 μM fluorodeoxyuridine, 10 μM cytosine arabinoside, 5 μM uridine, and 25 U/ml mycostatin. The cells were seeded on collagen-coated coverslips or collagen gels and kept in a humidified incubator with 5% CO_2/95% air at 37°C. All the experiments were performed with the cells in culture for 3–10 d.

Quick-Freeze, Freeze-Fracture, Deep-Etch EM of Cultured Chromaffin Cells

Cultured chromaffin cells on collagen gel (cell matrix typeI-A; Nitta Gelatin Co., Tokyo, Japan) were washed with Locke's solution (140 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 11 mM glucose, 0.546 mM ascorbate, 15 mM Hepes, 2 mM $CaCl_2$, pH 7.5) and the gel was excised and applied to an aluminum disk, quick-frozen, and deep-etched with a liquid helium-cooled machine as described previously (Heuser and Salpeter, 1979; Hirokawa and Heuser, 1981). For activation, Locke's solution containing 100 μM acetylcholine was added immediately before the freezing. Some cells were permeabilized with 20 μM digitonin in 70 mM KCl, 30 mM Hepes pH 7.2, 5 mM $MgCl_2$, 2 mM EGTA, 10 μM taxol, 1 mM PMSF, and 10 μM leupeptin for 10 min at room temperature before freezing (Nakata and Hirokawa, 1987). Replicas were made by rotary shadowing with platinum and carbon. Tissues were dissolved in chromic sulfuric acid overnight and cleaned with distilled water. Replicas were examined at 100 kV with a JEOL 1200EX electron microscope (JEOL USA, Peabody, MA).

Low-Angle Rotary Shadowing of Calpactin I Complex Molecule Alone

Calpactin I complex was purified from bovine lung by the method of Glenney et al. (1987). This purified calpactin I complex was suspended in 50% glycerol, 10 mM Tris, pH 7.0, 1 mM EGTA at a protein concentration of 100 μg/ml and then sprayed on freshly cleaved mica flakes as described previously (Tyler and Branton, 1980; Hirokawa, 1986). The samples were dried by vacuum evaporation and rotary shadowed with platinum in a freeze-fracture machine (model 301; Balzers, Furstentum, Liechtenstein) at an angle of 6°. The replicas were detached from the mica with hydrofluoric acid, washed with distilled water and collected on Formvar carbon-coated grids.

Quick-Freeze Deep-Etching of Calpactin I Complex Molecule Alone

The suspensions of 0.1 mg/ml purified calpactin I complex in 10 mM Tris, pH 7.0, 1 mM EGTA were dropped on mica flakes and quick-frozen (Heuser, 1983).

Calpactin Reconstitution Study In Vitro

Liposomes plus Calpactin I Complex. Liposomes were prepared by drying 1 mg phosphatidylserine plus 1 mg phosphatidylethanolamine plus 1 mg cholesterol (Sigma Chemical Co.) together and sonicating into a 70-mM KCl, 30-mM Hepes, 2-mM $MgCl_2$, pH 7.0 solution. To achieve OD_{320} = 0.3 (Glenney et al., 1987), the liposomes were diluted in a buffer (70 mM KCl, 30 mM Hepes, 2 mM $MgCl_2$, pH 7.0, EGTA/Ca buffer) containing 0.1 mg/ml calpactin I complex, and the free calcium concentration was adjusted (Portzehl et al., 1964). Liposome aggregation was monitored as an increase in turbidity (OD_{320}) and was observed with a Zeiss differential interference microscope (Carl Zeiss, Inc., Thornwood, NY) after 5 min incubation at 37°C (Glenney et al., 1987). Such liposome solutions were then applied to mica flakes and quick-frozen (Heuser, 1983). Each suspension of the same volume was centrifuged at 10,000 *g* for 15 min and the pelleted liposomes were analyzed by SDS-PAGE. As a control, liposomes in the same buffer without the protein were centrifuged and the pellets were quick-frozen.

Chromaffin Granules plus Calpactin I Complex. Chromaffin granules were purified from the bovine adrenal medulla by sucrose step gradient with 1 mM EGTA (Smith and Winkler, 1967). The isolated granules were mixed with the calpactin containing buffers with a concentration of 50 μM free calcium as described above to achieve OD_{540} = 0.3 (Durst and Creutz, 1988). After 5 min incubation at 37°C, this suspension was applied to mica flakes and quick-frozen.

Production and Purification of Anti-Calpactin I Heavy Chain 36K Antibody

Calpactin I was prepared from bovine small intestines according to the method of Gerke and Weber (1984). An outline of the production of calpactin I antibody was given elsewhere (Sobue et al., 1989). Antiserum against calpactin I was raised in New Zealand rabbits, and the anti-calpactin I antibody was purified from calpactin I antiserum by affinity chromatography using the calpactin I-conjugated Sepharose column.

Immunofluorescence of Calpactin I on Cultured Chromaffin Cells

Cells on a coverslip were washed with PBS and fixed in PBS-2% paraformaldehyde for 1 h at room temperature. After rinsing three times in PBS, the cells were permeabilized by incubating with 0.1% Triton X-100-PBS for 10 min. Some cells were processed further without Triton X-100 treatment. The cells were treated with 10% normal goat serum in PBS for 15 min at room temperature. Then the cells were incubated with affinity purified anti-calpactin I antibody diluted 1:20 with PBS, for 1 h at 37°C. The cells were again washed three times with PBS and incubated for 1 h at 37°C with

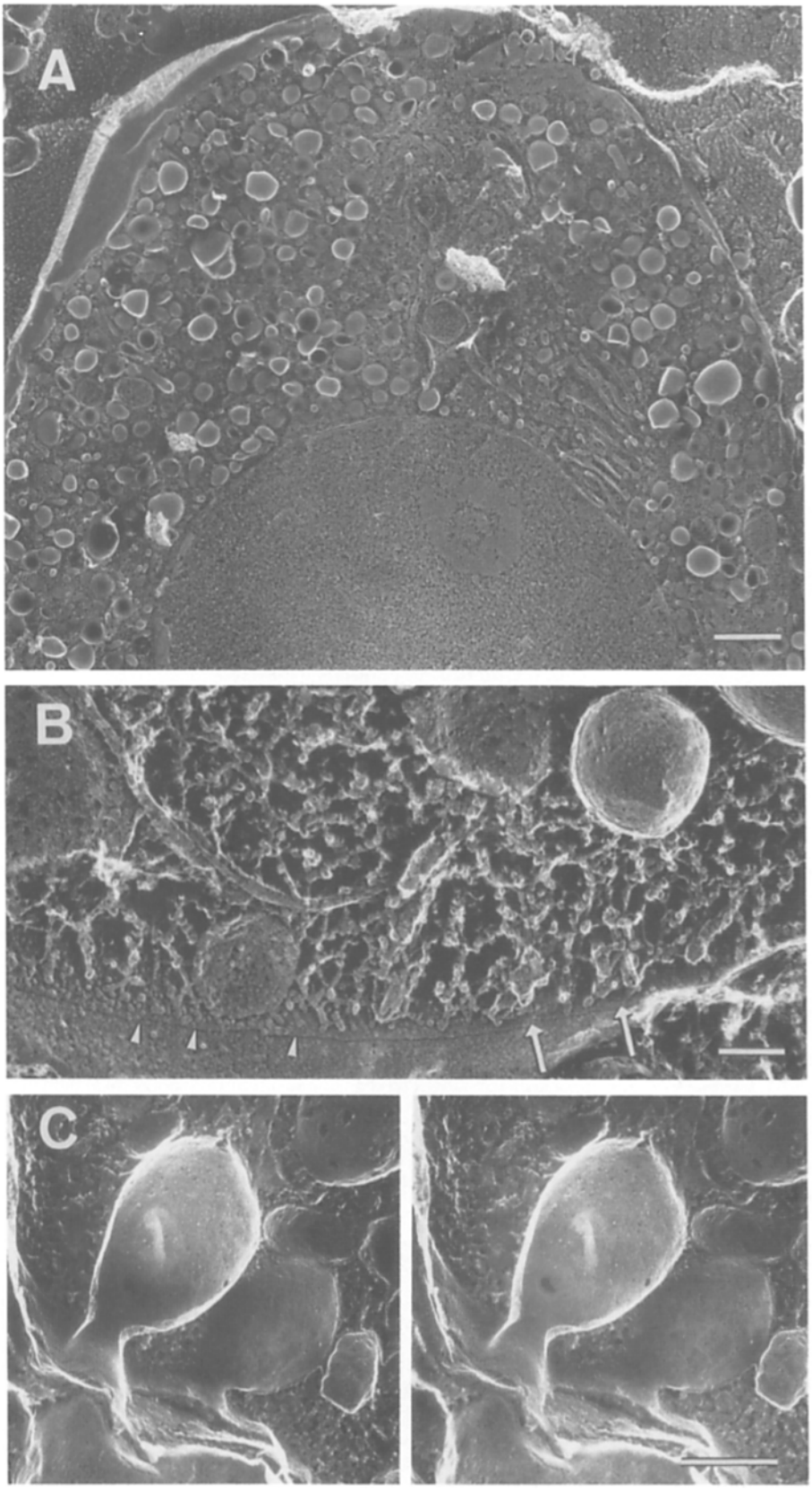


Figure 1. Quick-freeze, deep etch EM of cultured chromaffin cells. (*A*) Low magnification of fresh chromaffin cells. Cytoplasm is filled with vesicles except nucleus, Golgi, and ER systems (top right of the nucleus in the figure). Bar, 1 μm. (*B*) Subplasmamembranous area of the digitonin permeabilized chromaffin cell. The cortical cytoskeletal network and true inner surface of the plasma membrane are clearly shown. Note the small globular materials (*arrowheads*) on the inside of the plasma membrane. Some parts of the membane are rich in globules (*arrowheads*), and others are smooth (*arrows*). Bar, 0.1 μm. (*C*) 100 μM acetylcholine-activated chromaffin cell. Vesicles with the diameter of chromaffin granules fused with plasma membrane and formed Ω-shapes. Stereo pair. Bar, 0.1 μm.

1:100 diluted rhodamine-conjugated goat antibodies to rabbit IgG (Cappel Laboratories, Cochranville, PA). After vigorous washing in PBS, the cells were mounted in 70% glycerol-PBS.

Immunolocalization of Calpactin I on Frozen Ultrathin Sections

Chromaffin cells on collagen-coated dishes were washed with Locke's solution and activated with 100 μM acetylcholine for 60 s at 30°C and immediately fixed with 2% paraformaldehyde 0.1% glutaraldehyde PBS at 4°C for 1 h. For the unstimulated preparation, cells were washed and fixed without acetylcholine. Fixed cells were gently scraped off the dish with a razor and pelleted in 10% gelatin PBS. After cryoprotection by infiltrating with 2.3 M sucrose in 0.1 M phosphate buffer, the samples were frozen with liquid Freon 22. Ultrathin sections were cut by a cryo ultramicrotome at −90–100°C (UltraOmU4 equipped with FC-4; Reichert Jung, Vienna, Austria). The sections were picked up and attached to Formvar-coated grids. The sections were then stained for immunocytochemistry by the following procedure. After blocking with 10% normal goat serum, the sections were incubated with affinity-purified rabbit anti-calpactin I 36K (1:20) for 1 h at room temperature. They were washed extensively with PBS and incubated for 1 h with 1:20 diluted 5 nM colloidal gold-labeled goat anti-rabbit IgG (Janssen Life Science Products, Beerse, Belgium) in Tris-buffered saline, pH 8.2. After washing with the same buffer, the sections were fixed and mounted in methocell/acid uranyl (9:1) according to the method of Tokuyasu (1980). Nonspecific rabbit IgG instead of the first antibody was used as a control. For actin labeling, we used 1:1,000 diluted mouse mAb to actin (Amersham International, Amersham, UK) and 1:20 diluted 5 nm colloidal gold-conjugated rabbit IgG fraction of anti-mouse IgM (Janssen Life Science Products).

Other Methods

Protein concentration was determined by the method developed by Bradford (1976). Western blotting was performed as described by Towbin et al. (1979). SDS-PAGE was carried out using 12.5% running gel and 5% stacking gel according to Laemmli (1970).

Results

Vesicles Existed Just inside the Plasma Membrane and Connected to the Plasma Membrane via Short Strands in Cultured Chromaffin Cells after Stimulation

Cultured chromaffin cells were filled with chromaffin vesicles (Fig. 1 *A*). On stimulation with 100 μM acetylcholine, vesicles were fused with the plasma membrane and formed an exocytotic Ω-shape (Fig. 1 *C*). Although most of the vesicles were separated from the plasma membrane by cytoplasm, some were found in juxtaposition to the plasma membrane after stimulation. These vesicles were linked with the plasma membrane via very short (6–10 nm) strands (Fig. 2, *A–C, arrows*). We also noticed that numerous small globular materials with a diameter of ~10 nm existed on the cytoplasmic surface of the plasma membrane (Fig. 1 *B*). These globules were not evenly distributed over the plasma membrane, with some portions having an abundance of globules (*arrowheads*), and others being relatively bare (*arrows*).

Calpactin I Complex, a Globular Molecule with a Diameter of 10.7 nm, Is associated with Liposomes in a Calcium-dependent Manner

Calpactin I complex was purified from bovine lung (Fig. 3 *A*, lane *a*). Its single molecular structure was studied by low-angle rotary shadowing and quick-freeze, deep-etching on

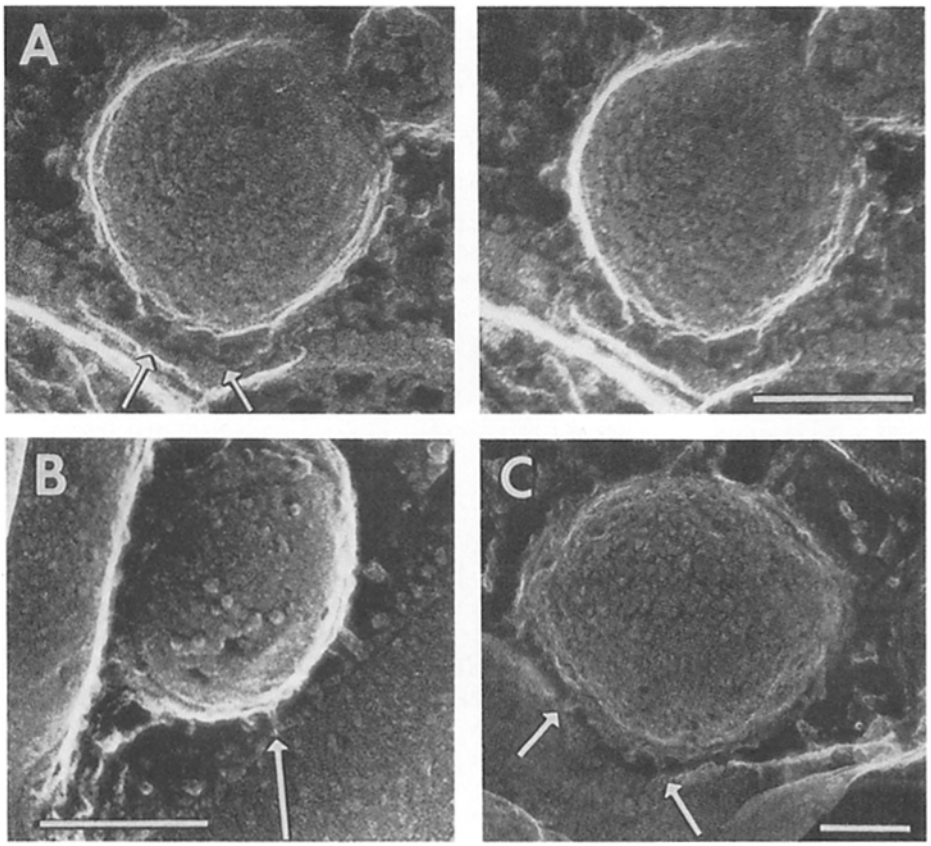


Figure 2. Vesicle- and plasma-membrane associations in cultured chromaffin cells. Some vesicles are closely associated with the plasma membrane (*A–C*), and very fine strands (*arrows*) cross-link these two membranes. (*A*) Stereo pair. Bar (*A–C*), 0.1 μm.

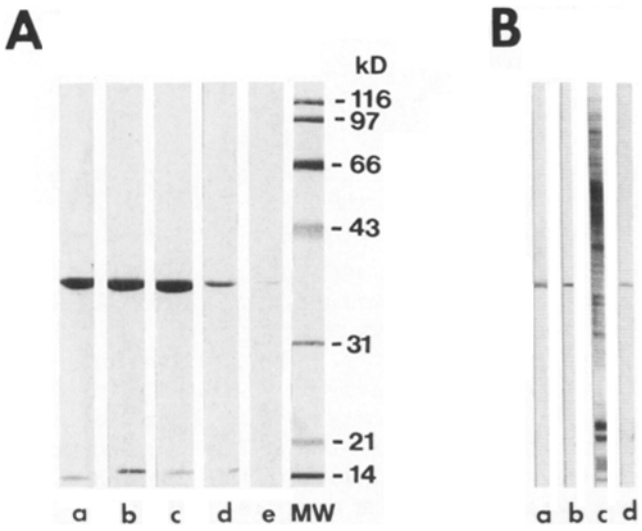


Figure 3. SDS-PAGE and Western blotting. (*A*) SDS-PAGE analysis of reconstitution study of calpactin plus liposomes. Lane *a*, purified calpactin I complex from bovine lung. Two bands corresponding to the 36K heavy chain and the 10K light chain are observed. Lanes *b–e*, calpactin I complex pelleted with liposomes by centrifugation at 5×10^{-5} M (lane *b*), 1×10^{-5} M (lane *c*), 1×10^{-6} M (lane *d*), and 1×10^{-7} M (lane *e*) free calcium concentrations. Lane (*MW*) Molecular mass standards. Note that the protein in pellets increased from 10^{-6} M, and reached a plateau at 5×10^{-5} M free Ca^{2+} concentration. (*B*) Immunoblotting of bovine calpactin I using affinity purified anti-calpactin I (36K) IgG. SDS-PAGE of purified bovine lung calpactin I complex (lane *a*), and bovine adrenal medulla whole homogenates (lane *c*) stained with amido black after the transfer to the nitrocellulose paper. Staining of the blots of purified protein (lane *b*), and the whole homogenates (lane *d*) with anti-calpactin antibody shows a single 36-kD band in both cases.

mica (Fig. 4). This molecule, which was seen to be globular in shape by both low-angle rotary shadowing method and the quick-freeze, deep-etch mica method, had a diameter of 10.7 ± 1.7 (SD) nm, (n = 489).

Purified calpactin I complex was mixed with phospholipid-containing liposomes at various free Ca^{2+} concentrations. The use of liposomes first instead of chromaffin granules in reconstitution studies had two advantages: (*a*) the possibility of the involvement of other membrane proteins can be ruled out, allowing us to focus on the interaction between calpactin and lipid membranes. (*b*) The surface of liposome membranes is very smooth, whereas the surface of isolated chromaffin granules is rough due to the presence of membrane association or integral proteins. Calpactin I molecules are more easily identified on the liposome membrane by quick-freezing. Calpactin I complex aggregated liposomes at $\geq 10^{-6}$ M free calcium concentration, which was monitored by OD_{320} elevation or directly observed by Nomarski differential interference microscopy (Glenney et al., 1987). Association of calpactin I complex with liposomes also increased at 10^{-6} M and reached a plateau at 5×10^{-5} M free calcium concentration by SDS gel analysis of the pelleted liposomes by centrifugation (Fig. 3 *A*, lanes *b–e*).

To obtain the maximum association of calpactin molecules with the membrane, liposomes were mixed with 0.1 mg/ml purified calpactin I complex at 50 μM free Ca concentration and quick-frozen on mica flakes. Many fine globules similar in size and shape to calpactin I complex on mica were observed on the surface of the aggregated liposomes in the presence of Ca^{2+} (Fig. 5, *A* and *B*), whereas no such globules were observed when only liposomes in the same buffer were quick-frozen (Fig. 5 *D*). Thus, these globules on the liposomes represent calpactin I complex molecules associated with lipid bilayers in a Ca^{2+}-dependent manner.

Although the same globules were found on liposomes in a calcium-chelated condition (1 mM EGTA), their numbers (2.0 ± 2.0 [SD] globules/[0.1 μm]²) were far less than those of the 50-μM Ca^{2+} concentration samples (25.2 ± 5.9 [SD]/[0.1 μm]²) and liposomes did not form aggregates (Fig. 5 *E*). The distinct difference in the number of calpactin molecules on the surface of liposomes between calcium-chelated and 50 μM free calcium conditions made it clear that globular calpactins on liposomes at 50 μM free Ca^{2+} do not represent the artifacts of etching in proteinous solutions.

Calpactin I Complex Cross-Links between Aggregated Liposomes and between Isolated Chromaffin Granule Membranes as Short Strands In Vitro

We found fine strands cross-linking between two facing surfaces of aggregated liposomes at 50 μM Ca^{2+} (Fig. 5 *A*, *arrowheads*). Fig. 5, *B* and *C* show higher magnifications of these fine strands, which are apparently different in shape from the globular structures on the free surface of the lipo-

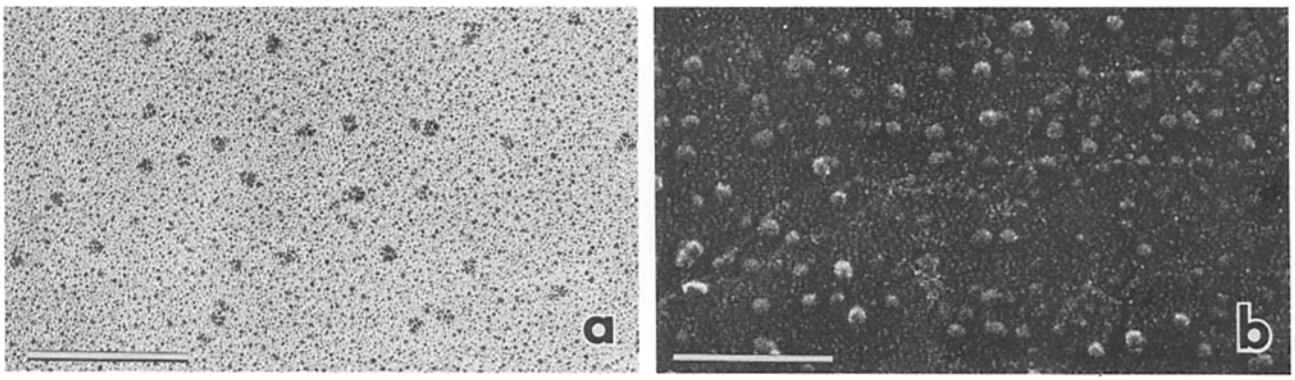


Figure 4. Molecular structure of the calpactin I complex alone on mica as revealed by low-angle rotary shadowing (*A*), and by quick-freeze, deep-etching on mica flakes. Both reveal globular structures. Diameter of the molecules was 10.7 ± 1.7 (SD) nm (*B*). Bars, 0.1 μm.

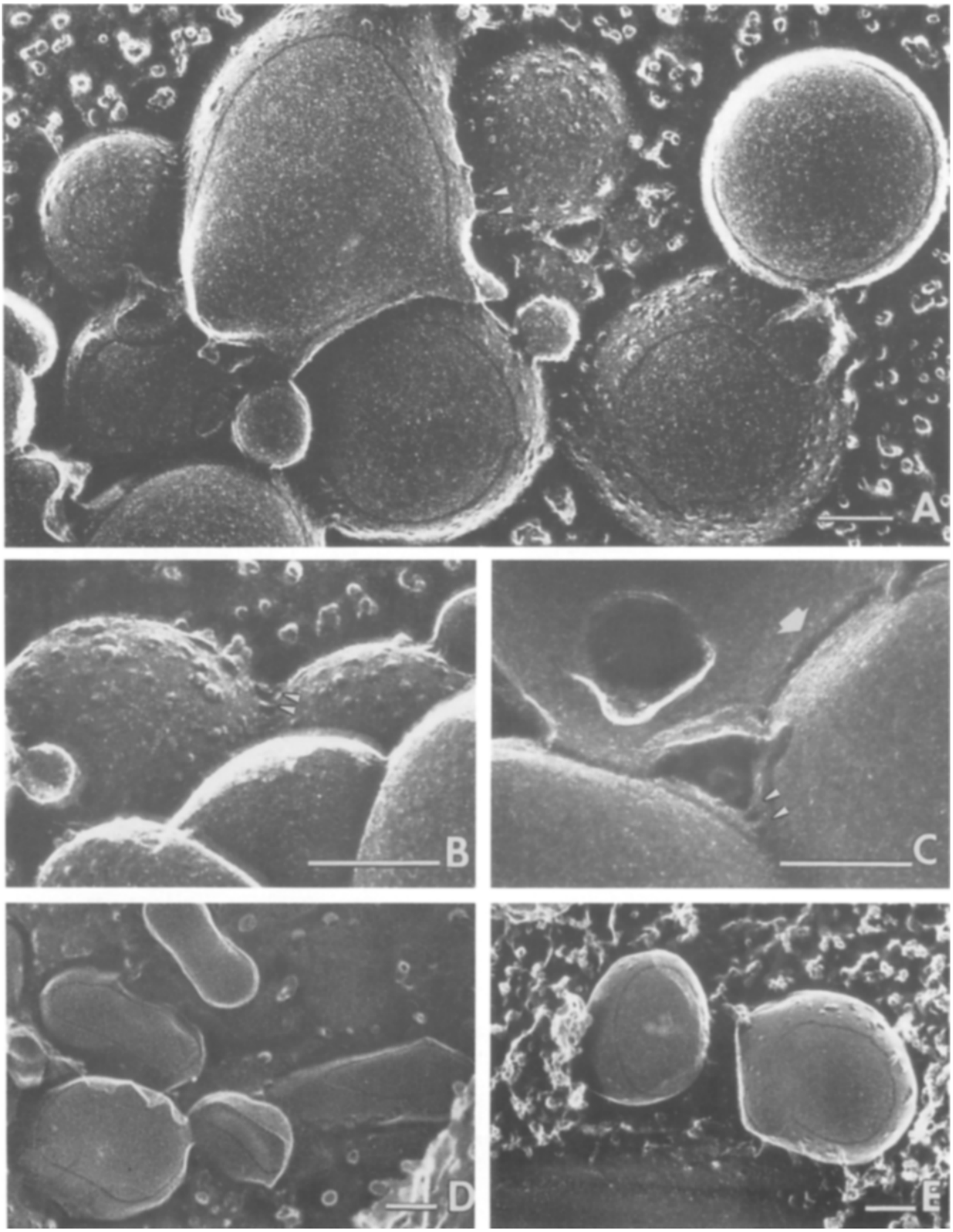


Figure 5. Calpactin reconstitution study with liposomes. At 50 μM free calcium concentration (*A–C*), liposomes were aggregated. Although lipid membranes were fractured between the bilayer, the true surface of the membrane was also observed by deep-etching. Numerous globular deposits were observed on the true surface of the liposomes (*A* and *B*). Cross-linking fine short strands were observed between the two aggregated membranes in (*A–C*) (*arrowheads*). *B* shows especially clearly the crosslinking structures, which are apparently different in shape from the globular calpactins. (*C*) On the fractured *E* face of the attached membrane of the aggregated liposomes, no transmembrane

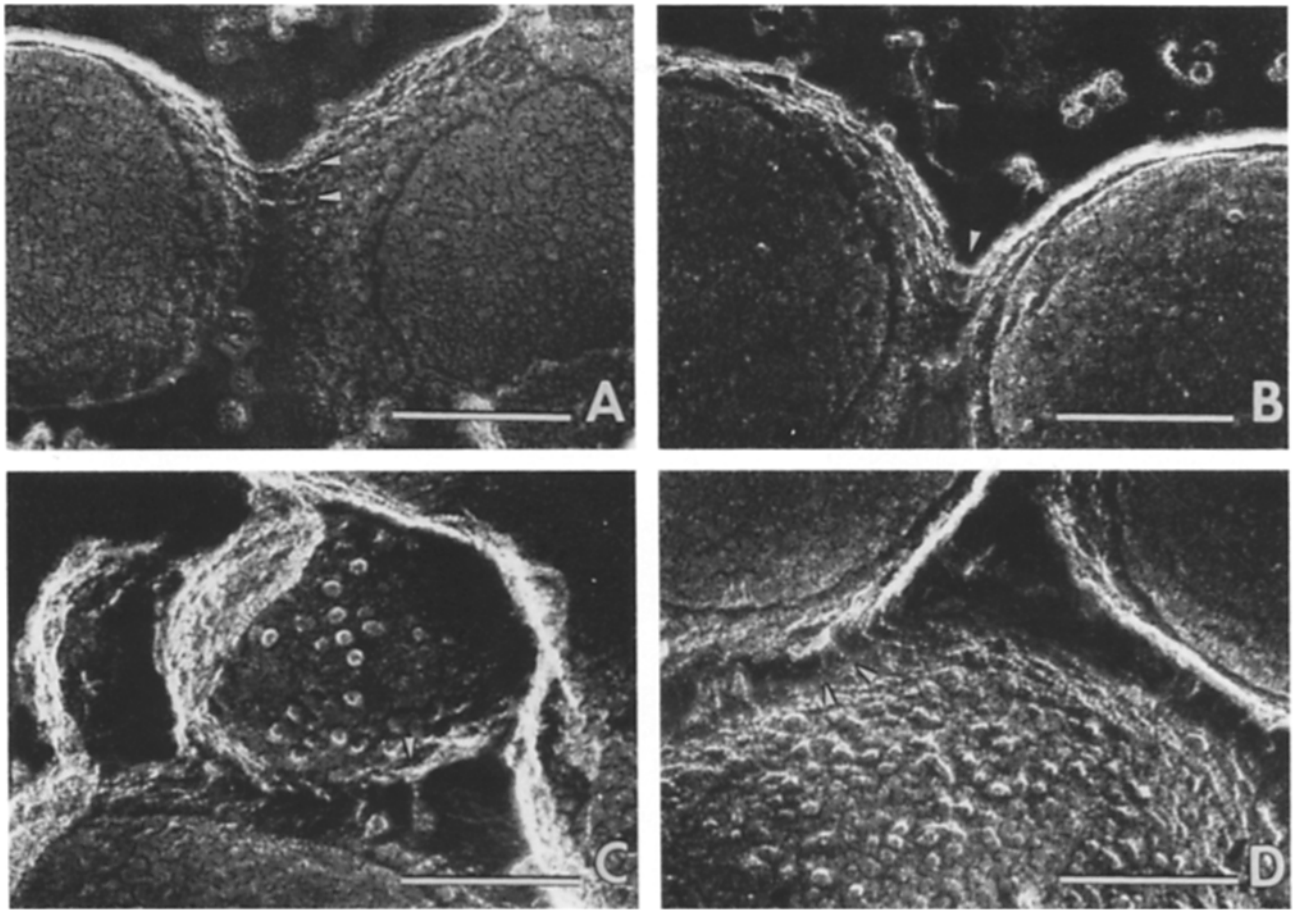


Figure 6. Calpactin reconstitution study with isolated chromaffin vesicles at 50 μM Ca^{2+} concentration. Vesicles were aggregated, and cross-linking fine strands were also observed (*A–D, arrowheads*). Bars, 0.1 μm.

somes. The average length of the strands was 6.5 $\pm$ 1.9 (SD) nm (n = 34). The fine strands might represent alternate forms of calpactin I complex molecules when they connect two phospholipid bilayers in the presence of Ca^{2+}, because only purified calpactin I complex and liposomes existed in this simple system.

Fig. 5 *D* is a control where liposomes were pelleted by centrifugation without calpactin molecules. Strand-like structures could not be observed between opposing liposomes. Furthermore, to eliminate the possibility that these fine strands might be the artifacts of deep-etching in the protein-containing solution, we performed quick-freezing on calpactin and liposomes under Ca^{2+}-chelated conditions. Although calpactins did not cause liposome aggregation (Fig. 5 *E*), some liposomes were attached to each other by chance. However, even in such cases, crosslinking structures were never observed.

Previous electrophysiological studies suggested that synexin, a calpactin-like molecule, forms transmembrane channels in phosphatidylserine membrane in the presence of calcium ions (Pollard et al., 1988). However, in the case of the calpactin molecule, no transmembrane particles were observed in the fractured surface of the attached membrane (Fig. 5 *C, arrow*).

We also examined the interaction between calpactin I complex and isolated bovine chromaffin vesicles instead of liposomes by quick-freezing on mica. When chromaffin vesicles were mixed with calpactin I complex, the vesicles aggregated in a calcium-dependent manner. We found similar cross-linking fine strands between the aggregated vesicles in the presence of Ca^{2+} (Fig. 6, *arrowheads*). The average length of these strands was 7.5 $\pm$ 2.2 (SD) nm (n = 49). In this case, the possibility does exist that some of the fine strands resulted from the interaction between calpactin and vesicle membrane proteins. In fact, some of the cross-linkers were pyramidal in shape (Fig. 6 *C*). However, judging from the length of these strands, many of them could be the result of the interaction between calpactin and lipid membrane, as observed in the liposome reconstitution study. These cross-linking fine strands, in size and shape, look very much like

structures were observed (*arrow*). Under 1 mM EGTA conditions (*E*), liposomes formed no aggregates and the fine globules on the surface of the liposomes are markedly decreased. (*D*) Liposomes in the same buffer without calpactin molecules were pelleted by centrifugation and quick-frozen as a control study. No globules and no cross-linking structures were observed. Bars in *A–E*, 0.1 μm.

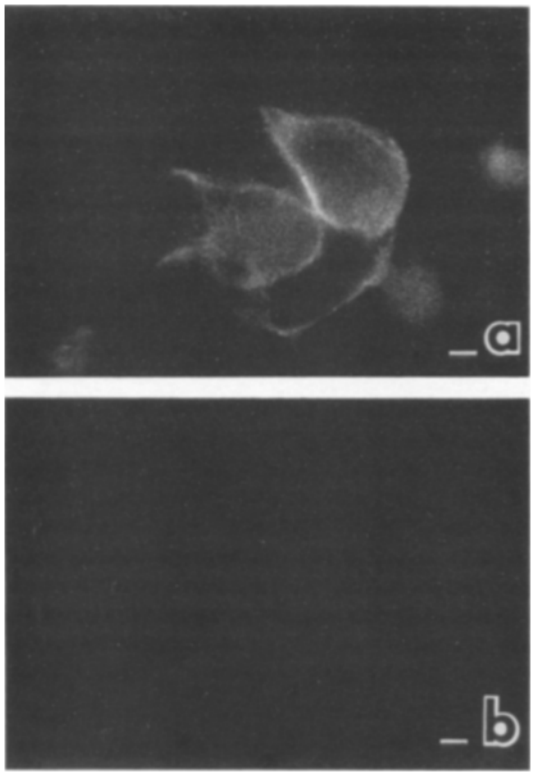


Figure 7. Immunofluorescence study of calpactin in cultured chromaffin cells. (*a*) Fixed and permeabilized cells. Cell periphery is stained. (*b*) Fixed but not permeabilized cells. No calpactin staining was observed. Bars, 1 μm.

those that we observed between chromaffin granules and the plasma membrane in cultured chromaffin cells (Fig. 2).

Calpactin I Was Closely Associated with the Inner Face of the Plasma Membrane of Cultured Chromaffin Cells

Affinity-purified antibody to calpactin I (p36) recognized the 36K subunit of the purified bovine lung calpactin I complex and the single band with the same molecular weight in whole cell homogenates of bovine adrenal medulla by Western blotting (Fig. 3 *B*). Calpactin I was localized mainly at the cell periphery of the cultured chromaffin cells by immunofluorescence (Fig. 7 *a*) as previously described (Burgoyne, 1988). Preincubation of the antiserum with purified calpactin I complex blocked this staining. At the electron microscopic level, calpactin I was localized mainly on the plasma membrane in both resting and activated chromaffin cells (Figs. 8 and 9, unstimulated samples not shown). In fact, the number of the gold labels on the plasma membranes was 8.8 ± 5.9 (SD)/μm along the membrane, whereas the number of the labels on the chromaffin vesicle membranes was 0.75 ± 0.46 (SD)/μm.

Careful observation of the plasma membrane labeling of chromaffin cells shows that a considerable portion of the gold particles appears to be localized on the exterior of the cell surface. However, no immunofluorescence could be detected when chromaffin cells were incubated with anti-p36 antibodies and probed with a fluorescein-conjugated second antibody without permeabilization (Fig. 7 *b*). In contrast, probing Triton X-100-permeabilized chromaffin cells with the same antibody solution clearly demonstrated high calpactin immunofluorescence in the cell periphery (Fig. 7 *a*). This result strongly suggests that calpactin I is located at the inner surface of the plasma membranes. It has been previously reported that gold particles appear to be localized at the outer cell surface when the localization of proteins existing at the inner surface was examined by the cryo ultrathin section method. The apparent localization of part of the gold particles on the outer cell surface must be due to "plane of section" artifacts (Boonstra et al., 1985; VanHooff et al., 1989) or to the extension of the antibody gold complex. In fact, most of the gold labels were observed inside the cells when the plasma membrane was sectioned en face (Fig. 8 *c*), or two plasma membranes were faced with each other (Fig. 9 *c*). In both cases, the artifact that occurs at the edge of the sections is expected to be avoided. The presence of calpactin at the inner surface of other cells has been suggested at the light microscopic level (Greenberg and Edelman, 1983).

To demonstrate how closely calpactin molecules are associated with the inner surface of the plasma membrane, we compared the localization of calpactin I (Figs. 8 and 9) with that of actin (Fig. 10) on frozen ultrathin sections. In chromaffin cells, actin forms a cytoskeletal network in the cell periphery (Fig. 1 *B*). By immunofluorescence, we cannot detect the difference in the localization of calpactin and actin (Burgoyne and Cheek, 1987). Comparison of the localization of the gold particles in Figs. 8 and 9 clearly shows that calpactin is more closely associated with the plasma membrane than peripheral actin networks.

Calpactin was not distributed evenly over the plasma membrane. Its distribution was rather patchy (Fig. 8, *a* and *b*), just like the globules observed inside plasma membranes by quick-freezing (Fig. 1 *B*). Few gold labels were observed on other membrane organelles such as the nuclear membrane and ER systems (Fig. 8 *a*).

Calpactin I Exists between the Plasma Membrane and the Facing Surface of the Chromaffin Vesicles after Activation

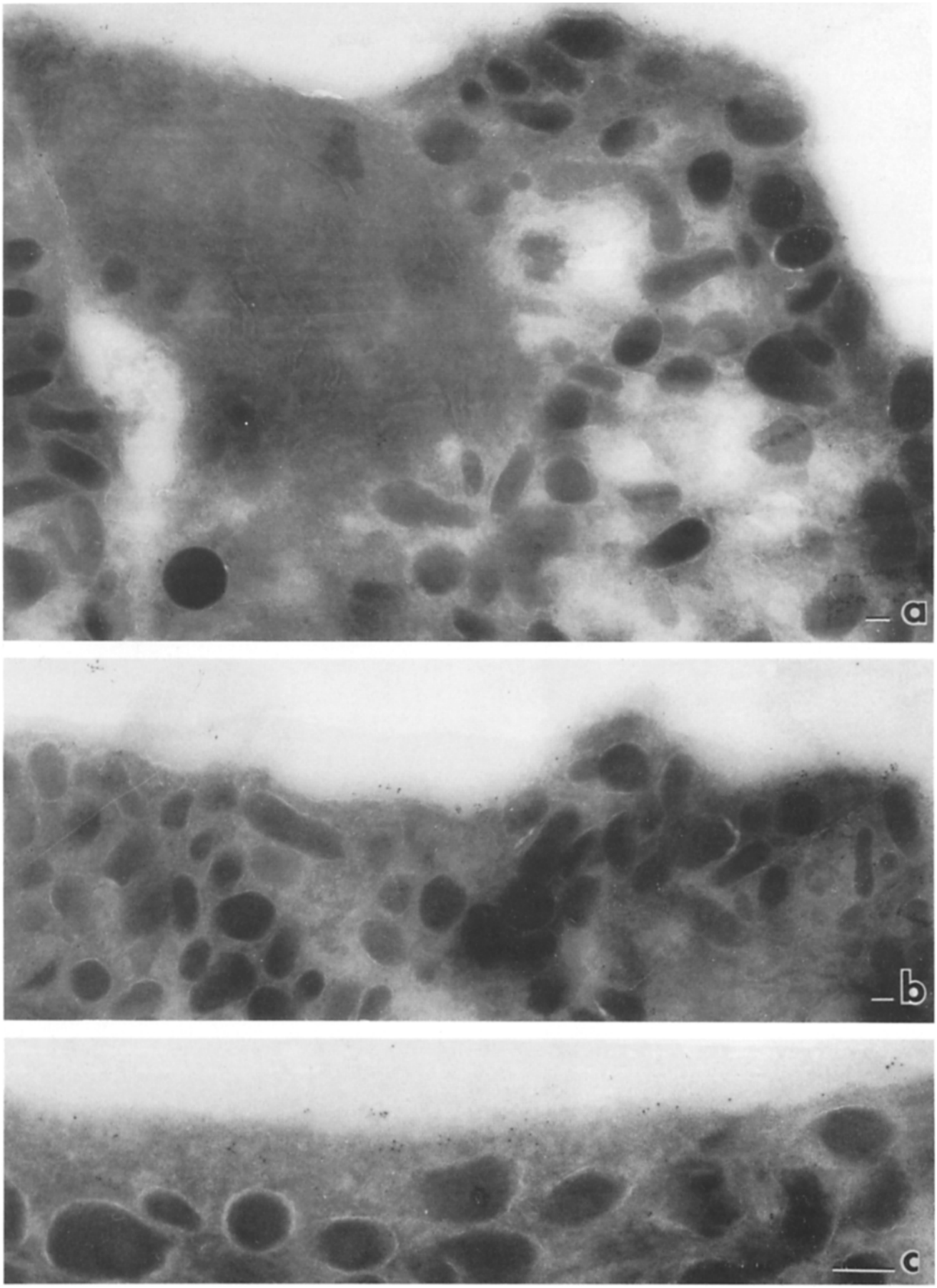
In activated cells with acetylcholine, some of the chromaffin vesicles were attached to the plasma membrane. In such a case, gold labels were frequently seen to be closely associated with the attached sites. Fig. 9, *d–h* shows typical examples of calpactin localization at the attaching sites of the plasma membrane and chromaffin vesicles.

Discussion

Two Forms of Calpactin I Complex Molecules Associated with Lipid Membranes

We observed fine strands (6.5 ± 1.9 [SD] nm long) connect-

Figure 8. Localization of calpactin on cryo ultrathin sections of activated cultured chromaffin cells at the electron microscopic level. 5 nm colloidal gold particles were mainly localized on the plasma membrane. Careful observation revealed its distribution to be patchy (*a* and *b*). Few labels were observed on the other membrane structures (*a*). In (*c*), the plasma membrane was sectioned en face. Bars, 0.1 μm.


a
b
c

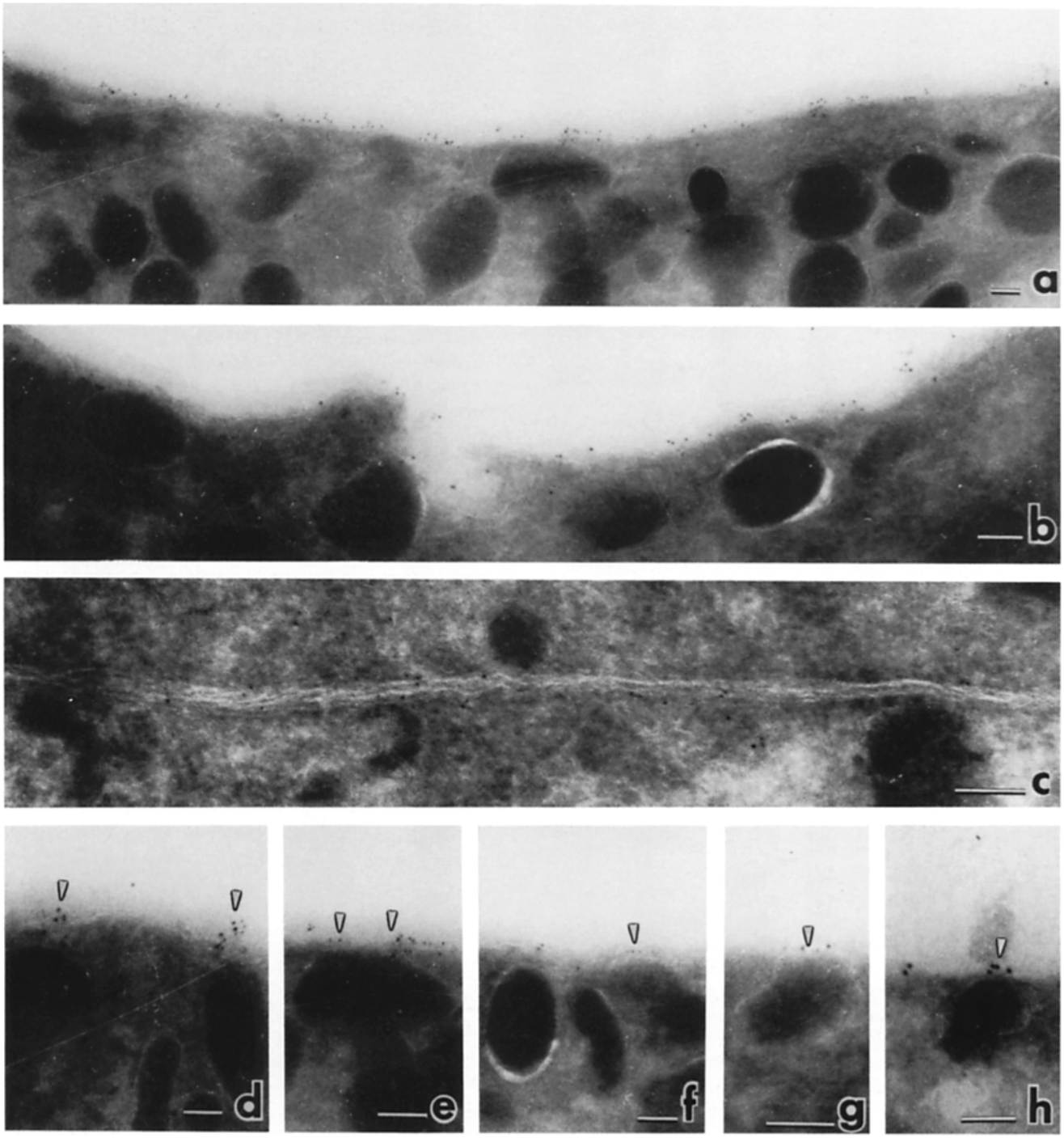


Figure 9. Localization of calpactin on cryo ultrathin sections of activated cultured chromaffin cells at the electron microscopic level. (*a–c*) Most of the labels were on the plasma membrane. Gold labels tend to drop down the section (*a*) at its edge, while most of the labels were observed just inside the plasma membranes at cell-to-cell contact face (*c*). (*d–h*) Gallery of the vesicle-plasma membrane associations. *d*, *e*, and *f* are higher magnifications of parts of Fig. 8 *b*, Fig. 9 *a*, and Fig. 8 *a*, respectively. Note the 5-nm gold particles closely associated with the vesicle-attaching sites on the plasma membrane (*arrowheads*). Bars in *a–h*, 0.1 μm.

ing the adjacent two liposomes in addition to the globular structures in reconstitution studies at 50 μM Ca^{2+}. We proposed that these fine strands were also calpactin molecules having undergone conformational change.

Previous biochemical data and the cDNA sequence study of calpactin I have shown the following features of the calpactin I complex. The calpactin light chain (10K) is known to exist as a dimer (Gerke and Weber, 1985). The heavy chain (36K) consists of two distinct domains, a 33-kD core domain and a 3-kD tail (Glenney and Tacks, 1985). The 33-kD core domain contains two Ca^{2+}/phospholipid-binding sites (Glenney, 1986; Glenney et al., 1987; Johnsson et al., 1986; Saris et al., 1986). The 3-kD tail domain interacts with the light chain (Glenney, 1986*b*; Glenney et al., 1986). Thus, a

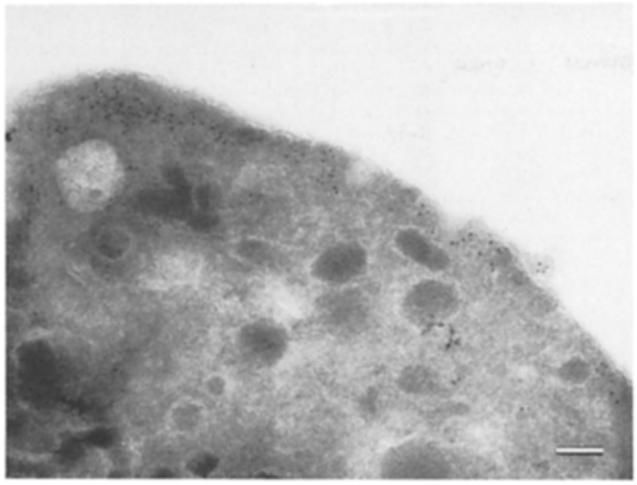


Figure 10. Localization of actin on cryo ultrathin sections of unstimulated chromaffin cells at the electron microscopic level. 5 nm colloidal gold particles are located in the subplasmamembranous area as a band. Chromaffin vesicles existed inside this band. Bar, 0.1 μm.

model was proposed that two light chains intervene between two heavy chains (Glenney et al., 1987). Because the fine strands were observed only between two liposomes and the molecules took globular shape in other places even in the presence of Ca^{2+}, it can be assumed that not only Ca^{2+} and phospholipid but also some mechanical tension would be required for the formation of fine strands. In such cases, each 33-kD domain of a calpactin heterotetramer would bind to each liposome and the molecule would be stretched by the tension of both lipid membranes, whereas a globular shape would be a mechanically stable form. Calcium-dependent conformational change in the 36K subunits was detected by a change in UV absorption (Gerke and Weber, 1985). The possibility must be entertained that this conformational change in the heavy chain was also, at least in part, the cause of the observed shape change of the calpactin molecule.

The size of the globule on the mica and lipid membrane is consistent with one calpactin I complex $(36K)_2(10K)_2$ by our method, in comparison to other globular molecules (Heuser, 1983; Hirokawa and Hisanaga, 1987). As a protein with molecular weight of ~80,000 would be larger than a 6.5-nm rod (Hirokawa et al., 1989), perhaps a portion of the protein is embedded in each of the two connected membranes.

Possible Roles of Calpactin I Complex in Exocytosis

In this study, we found fine short strands (6–10 nm) cross-linking between the plasma membrane and the chromaffin vesicles after stimulation by quick-freeze, deep-etching (Fig. 2). We also found some globular structures (~10 nm) on the inner face of the plasma membrane (Fig. 1 *B*). Calpactin I appeared to be a good candidate for these strands from its chemical properties to aggregate lipid membranes at physiological micromolar order Ca^{2+} concentrations (Fig. 3). We found cross-linking short strands (6.5 ± 1.9 nm) besides the globular structures (~10 nm) in calpactin reconstitution studies in vitro (Figs. 4–6). Furthermore, EM immunocytochemistry showed that calpactin was very closely associated with the inner face of the plasma membrane (Figs. 7–9). Calpactin was especially seen to be located at the attaching sites between the plasma membrane and chromaffin vesicles after activation (Fig. 9, *d–h*).

Quick-freeze, freeze-substitution studies on *Limulus* amebocytes showed dense, elongated structures (5–8 nm diameter) between the plasma membrane and the facing surface of the granule membrane (Ornberg and Reese, 1981). Freeze-fracture studies showed connections (9 ± 1 nm) between the plasma membrane and chromaffin granules after stimulation. We demonstrated the details of the fine strands (6–10 nm) between the plasma membrane and chromaffin granules in stimulated, cultured chromaffin cells by quick-freeze deep-etching (Fig. 2). These connections were observed more frequently in stimulated cells, and most of the chromaffin vesicles were separated from the plasma membrane by cytoplasm in the unstimulated cells (Fig. 10) (Geisow and Burgoyne, 1987).

Immunocytochemistry revealed that calpactin I is closely associated with the plasma membrane and is also located between the plasma membrane and the facing surface of the chromaffin vesicles after activation (Figs. 7–9). Then, what structures in quick-freeze replicas (Fig. 1, *B,2*) could correspond to the calpactin molecules? The single molecular structures (Fig. 4) suggested that some of the globular structures (~10 nm) on the inner face of the plasma membrane would indeed represent calpactin molecules. Also, some of the fine strands (6–10 nm) between the plasma membrane and the chromaffin vesicles after activation might very well correspond to the fine strands observed in reconstitution studies (Figs. 5 and 6). As discussed before, it is quite plausible that some of the fine strands would be the result of the interaction between calpactin molecules and other membrane proteins in the reconstitution study with isolated chromaffin vesicles. This might explain why fine strands observed in the activated cells vary in detail. However, we observed the same strands between liposomes incubated with

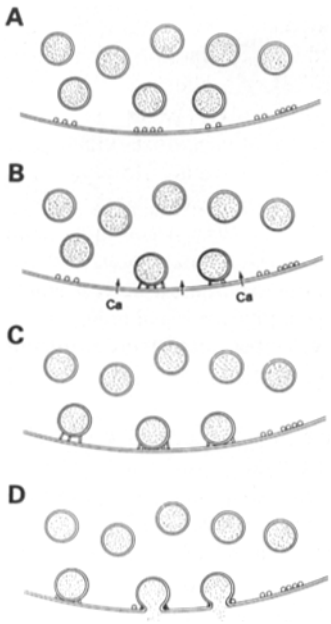


Figure 11. A hypothesis of calpactin involved in exocytosis in chromaffin cells. At a resting state, calpactin would exist as globules on the inner face of the plasma membrane (*A*). On activation followed by Ca^{2+} influx, calpactin would change its shape to cross-link the nearby secretory vesicles (*B*). Vesicles would be tightly attached to the plasma membrane by calpactin (*C*). Fusion of the two membranes occurs and the vesicle contents are released (*D*).

calpactin at elevated Ca^{2+} concentrations. Yet, as there are no membrane proteins in liposomes, this result strongly suggested that calpactin alone could form fine strands. Even though other vesicle membrane proteins are involved in the case of chromaffin vesicles, the salient fact is that the fine strands, as observed in the cells, were found between the membranes only when chromaffin vesicles were aggregated by calpactin in the reconstitution study. Another notion of some likelihood may be that some of the fine strands in the cell (Fig. 2) are made of different chemical components, such as GTP-binding protein and receptor complex (Bourne, 1988). In any case, our present results indicated that calpactin molecule, from its chemical nature, from its molecular structure and from its localization, would appear to be a leading candidate for at least some of the fine strands between the plasma membrane and chromaffin vesicles after activation of the chromaffin cell.

From these findings, the following hypothesis could be postulated (Fig. 11). In a resting state, calpactin molecules would be mainly associated with the plasma membrane as fine globules. Cell stimulation is followed by Ca^{2+} influx, which elevates Ca^{2+} concentration to micromolar levels in the cell periphery. This would cause activation of the calpactin I complex on the plasma membrane. The cross-linking of the nearby vesicles and the plasma membrane would occur with a conformational change of the connecting calpactin I complex molecule. The chromaffin vesicles would be tightly attached to the plasma membrane by Ca^{2+}-activated calpactin molecules. This docking would be followed by the fusion of two membranes. The mechanisms are unknown, but involvement of arachidonic acids has been suggested (Creutz, 1981; Durst and Creutz, 1988). Calpactin might not act on secretory vesicles at a greater distance from the plasma membrane, judging from its close association with the plasma membrane. The process of how the vesicles get to the membrane remains unknown. In synapses it has been suggested that cytoskeletal molecules such as synapsin I and actin may be involved in the process (Llinas et al., 1985; Landis et al., 1988; Hirokawa et al., 1989). Calpactin might enable the membranes to come close enough to fuse by connecting the membranes more tightly upon Ca^{2+} entry.

Calpactin inhibits phospholipase A_2 by coating the substrates through its Ca^{2+}-dependent interaction with phospholipids (Davidson et al., 1987). An alternative possible role of calpactin is that calpactin regulates exocytosis by inhibiting the fusogenic process so that exocytosis may occur properly and excess fusions may not occur. However, this case is unlikely, because aggregated chromaffin granules by calpactin easily fused with each other when arachidonic acid was added in reconstitution studies (Durst and Creutz, 1988). In any case, localization of the molecule, which interacts strongly with the membrane upon physiological elevation of Ca^{2+} concentrations at exocytotic sites, implicates calpactin as a potent regulator of exocytotic events in cultured chromaffin cells.

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