LIPID AND PROTEIN INTERACTIONS IN Ca^{2+}-PROMOTED AGGREGATION AND FUSION OF CHROMAFFIN GRANULE MEMBRANES

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The membranes of secretory granules are especially designed for the recognition of the cytoplasmic surface of the plasma membrane and for Ca^{2+}-triggered fusion with the latter to bring about the exocytotic release of the vesicular contents from the cell. Isolated bovine adrenal medullary chromaffin granule and *Torpedo* electric organ synaptic vesicle membranes have been shown to be capable of highly specific particle-particle recognition, aggregation, and fusion reactions, and may serve as a model for exocytosis (1–6). A hypothetical reaction scheme for these processes is presented in Fig. 1. Aggregation (reactions *a–c*) may be studied by light-scattering techniques (6); molecular rearrangements leading to fusion can be followed by suitably placed fluorophores using fluorescence resonant energy transfer (7, 8).

RESULTS AND DISCUSSION

Experiments with extracted chromaffin granule phospholipids show that aggregation cannot be explained solely on the basis of the characteristics of phospholipid bilayer regions of the membrane. Chromaffin granules have the following phospholipid composition: ~ 27% (mol/mol) phosphatidylcholine (PC); ~ 17% lyso-phosphatidylcholine (LPC); 34% phosphatidylethanolamine (PE) and ~ 8% phosphatidylserine (PS) (9). Vesicles composed of pure PC cannot be induced to aggregate by ionic perturbation; repulsive interaction between PC bilayers has been well documented. Vesicles composed of pure PS can be induced to aggregate by millimolar concentrations of divalent cations. We have proposed that the divalent cations form salt bridges between the charged head groups of the two membranes (10). Vesicles composed of pure PE aggregate at neutral pH by a cation-independent reaction. This may be the result of hydrogen bonding of PE head groups between membranes (11). Dilution of either PE or PS with PC greatly decreases the ability of the vesicles to aggregate (10–11). Vesicles prepared from extracted chromaffin granule lipids can be induced to aggregate by divalent cations. Aggregation (dimerization) can also be induced by elevating the monovalent cation concentration. Analysis of the aggregation kinetics by stopped-flow rapid mixing experiments shows that all of the phospholipid vesicle aggregation processes occur at rates which are approximately two orders of magnitude lower than the limit predicted by diffusion control (3). This is due to the influence of significant activation energy barriers to aggregation and to the necessity of rearrangements in the polar head group region for the formation of stable aggregates (4).

In contrast, the aggregation reactions of granule membranes occur at diffusion-controlled rates, indicating that energy barriers are absent and slow membrane rearrangements are not necessary. Arrhenius plots of the rates show a break at ~ 7°C, which correlates with membrane structural changes observed by other methods (3, 12). Furthermore, dimerization can be induced by monovalent

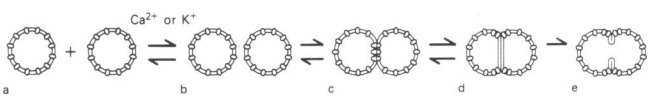

FIGURE 1 Mixed granule membranes, *a*, form encounter complexes, *b*, and stable complexes, *c*. The proteins and lipids undergo rearrangements allowing close membrane apposition, *d* and eventual fusion, *e*. K^+ and Ca^{2+} promote reactions *a–d*. Fusion requires Ca^{2+}. (*c.f.*, Fig. 4 of reference 4).

cations at diffusion-controlled rates (3). These results lead to the conclusion that the primary step in aggregation of secretion granules is the establishment of contact by membrane proteins at points several tens of Ångströms from the membrane surface. These proteins would be mirror-image symmetrically distributed on the membrane surface and only a small number need make transmembrane contact to form a stable complex. Further stabilization is made possible by the establishment of contact between phospholipid regions. This occurs by lateral diffusion of PS and PE into the region of contact and diffusion of PC away from this region (4).

Recent thin-section and rapid-freeze/freeze-fracture electron micrographs of aggregated membranes (7, 8) confirm earlier results (13) which showed that integral proteins are excluded from contact regions. This indicates that there are lateral as well as transmembrane protein-protein interactions involved in the structural changes.

This analysis is supported and extended by fluorescence energy transfer experiments in which the proteins of the membranes have been specifically labeled *in situ* using maleimide and mercury acetate derivatives. These reagents couple to free SH-groups. Protein-free lipid extracts of labeled membranes contain virtually no label. Because of the relatively small increases in acceptor fluorescence and the artifactual increase in fluorescence due to particle aggregation, the quenching of donor fluorescence was followed as an unambiguous signal of probe interaction.

When the donor and acceptor were placed on proteins of the same membrane, Ca^{2+} increased donor quenching with a K_m of ~ 200 μM, a concentration too low to promote significant aggregation. The effect was also independent of membrane concentration, showing that it must be the result of intramembrane protein clustering or patching. This constitutes the first demonstration of the fluid mosaic nature of a subcellular organelle.

If the donor and acceptor labels are placed on separate membranes, mixing in the absence of Ca^{2+} produces a slow, essentially zero-order decline in donor fluorescence. This effect can be greatly reduced by extensively washing the membranes and is ascribed to exchange of membrane proteins either through the medium or by granule-granule collisions (14). If the two sets of labeled membranes are mixed and Ca^{2+} added immediately or if the mixture is incubated overnight to allow for complete protein exchange, addition of Ca^{2+} produces a protein concentration-dependent increase in donor quenching with a K_m of ~ 2 mM and rates 5 to 10 times slower than Ca^{2+}-promoted aggregation (K_m ~ 4 mM) measured in parallel. We attribute this fluorescence change to slow rearrangements of membrane components which follow aggregation.

No component with rates similar to aggregation is seen by this labeling method. Since Ca^{2+} aggregates labeled or unlabeled granules at the same rate, this implies that the protein(s) responsible for granule-granule recognition either contain no free sulfhydryl groups or the labeled sites are far enough apart when the proteins interact for no significant Förster energy transfer to occur.

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