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Proteins on exocytic vesicles mediate calcium-triggered fusion

(secretion/sea urchin egg/fertilization/exocytosis)

STEVEN S. VOGEL AND JOSHUA ZIMMERBERG

Laboratory of Theoretical and Physical Biology, National Institute of Child Health and Human Development, and Laboratory of Biochemistry and Metabolism, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892

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ABSTRACT **In many exocytic systems, micromolar concentrations of intracellular Ca^{2+} trigger fusion. We find that aggregates of secretory granules isolated from sea urchin eggs fuse together when perfused with ≥10 μM free Ca^{2+}. Mixing of membrane components was demonstrated by transfer of fluorescent lipophilic dye, and melding of granule contents was seen with differential interference microscopy. A technique based upon light scattering was developed to conveniently detect fusion. Two protein modifiers, trypsin and *N*-ethylmaleimide, inhibit granule–granule fusion at concentrations similar to those that inhibit granule–plasma membrane fusion. We suggest that molecular machinery sufficient for Ca^{2+}-triggered fusion resides on secretory granules as purified and that at least some of these essential components are proteinaceous.**

Membrane fusion is a fundamental process by which exocytic secretion, enveloped viral entry, intracellular trafficking, and fertilization occur. Although viral fusion proteins have been rigorously identified (1), exocytic fusion proteins remain unidentified. Unambiguous identification of exocytic fusion proteins has been hampered by the lack of an efficient assay *in vitro* for membrane fusion.

As a first step in identifying fusion proteins, we wish to determine where these proteins reside. Because the Ca^{2+}-triggered fusion of sea urchin cortical granules with the egg plasma membrane (PM) does not require cytoplasmic proteins or factors (2–6), it is an ideal system for studying membrane proteins needed for fusion. The echinoderm oolemma is lined with a monolayer of about 15,000 cortical granules whose simultaneous exocytosis during fertilization results in the raising of the fertilization envelope, a block to polyspermy. Agents that mimic sperm by increasing intracellular Ca^{2+} also cause cortical granule fusion (7). A preparation consisting primarily of PM and cortical granules can be prepared quite easily (8). Such purified cortices react to micromolar Ca^{2+} with vectorial discharge of granule contents into defined ionic solutions (4, 5, 9). Cortical granules, purified from planar cortices, will bind to the cytoplasmic surface of the egg PM and then fuse with vectorial discharge of granule contents upon addition of Ca^{2+} (10, 11). We take advantage of the ability to reconstitute exocytosis to determine whether molecular components that purify with the cortical granules are sufficient to allow Ca^{2+} to trigger fusion.

METHODS

Preparation of Cortical Granules. *Strongylocentrotus purpuratus* adults were purchased from Marinus (Long Beach, CA). Eggs were prepared as described (5). Cortical granules were isolated as follows. Eggs were attached to the bottoms of plastic tissue culture flasks (175 cm^2) that had been treated for 3 min with 0.25 mg of poly(D-lysine) M_r > 300,000, Sigma). Unattached eggs were removed by washing with PKME (50 mM Pipes, pH 6.7/425 mM KCl, 10 mM $MgCl_2$/5 mM EGTA). Immobilized eggs were lysed in PKME by hitting the sides of flasks. After several PKME washes to remove free cytoplasm and loosely attached vesicles, egg cortices remained attached to the plastic flask. Granules were removed from these cortices by incubation in KEA (450 mM KCl/5 mM EGTA/50 mM NH_4Cl, pH 9.1) and again hitting the sides of flasks. Granules were pelleted at 1000 × *g* for 10 min at 4°C and resuspended in 1 ml of KEA per flask (≈0.2 mg/ml). In some experiments granules were further purified by passing them through 10-μm Nitex mesh and glass wool pretreated with 0.25 mg of poly(D-lysine) per ml. This treatment did not alter their ability to fuse with each other. Isolated granules were kept on ice until ready to use. Microscopic examination of the material revealed only individual cortical granules. Prior to use, the pH of the cortical granules was adjusted to pH 6.7 by the addition of 1 M Pipes (pH 6.1). Microscopic examination of the preparation at this point shows only isolated and clumped cortical granules.

Video Microscopy. Cortical granules were centrifuged onto fragments of glass coverslips (no. 0 thickness) and then transferred into a perfusion chamber. Microscopic examination at this time primarily revealed clumps of cortical granules adhering to glass. Occasionally, we saw amorphous material above the granules. However, the presence of this material (presumably formed from lysed granules) did not affect the results. The methods for differential interference microscopy (DIC), video microscopy, and imaging have been described (12) except that a 1.4-n.a. condenser was used. Free Ca^{2+} concentrations ≥ 1 μM were measured by using a Ca^{2+} electrode (13) calibrated with defined reference buffers (14). Ca^{2+} concentrations < 1 μM were calculated by using the algorithm of Fabiato and Fabiato (15).

Fluorescent Microscopy. Cortical granules were centrifuged onto coverslips and then transferred into a perfusion chamber. Granules were stained by perfusion with 1–3 ml of a 1 μM micellar mixture of the fluorescent cationic membrane probe 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (Di-I; Molecular Probes) in PKME buffer that was prepared immediately before use from a 100 μM stock solution of Di-I in ethanol. Presumably, incorporation of Di-I micelles into individual granules occurred upon collision. This led to randomly stained granules within clumps of unstained granules. The slide was next perfused with PKME to remove nonincorporated Di-I micelles. Neither 165 μM propyl gallate nor 1 mM hydroquinone interfered with Ca^{2+}-triggered fusion.

Light-Scattering Assays. Suspensions of cortical granules (100 μl per 0.32-cm^2 microtiter-plate well) were mixed with 100 μl of PKME solutions containing various Ca^{2+} concentrations (pH 6.7). The resulting turbidity of the granules in

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Abbreviations: DIC, differential interference microscopy; PM, plasma membrane; CG–CG fusion (contact), cortical granule-to-cortical granule fusion (contact); CG–PM fusion, cortical granule-to-PM fusion; Di-I, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate.

solution, measured in optical density (OD) units at 405 nm was determined on a microtiter plate reader (ICN/Flow Laboratories). In the absence of Ca^{2+}, the turbidity of the cortical granules was typically 0.1, unless otherwise noted. To measure the light scattering of contacting, layered granules, the microtiter plates containing the suspended cortical granules at various Ca^{2+} concentrations were centrifuged (1000 × *g* for 5 min), and the turbidity of the resulting granule sheets and fused granules was measured.

The Ca^{2+} dependence of cortical granule-to-cortical granule (CG–CG) fusion was determined by mixing 100 μl of granules in PKME with 100-μl aliquots of PKME solution containing various Ca^{2+} concentrations. Mixing did not change pH. The granules were pelleted to maximize CG–CG contact, and the turbidity at 405 nm was recorded for each well (≈0.2 in the absence of Ca^{2+}). The extent of exocytosis in isolated planar cortices was measured as described (5).

Trypsin and *N*-Ethylmaleimide (NEM) Inhibition. Cortical granules were centrifuged to the bottom of 96-well titer dishes. Granules were then incubated for 1 hr at room temperature with various concentrations of trypsin or for 30 min with various concentrations of NEM, which was prepared immediately prior to use. Subsequently, an equal volume of either PKME or PKME with Ca^{2+} (final free Ca^{2+} ≈350 μM; pH 6.7) was added to each point. The samples were again centrifuged. The turbidity was then measured for each reaction point. Because there was a small decrease in turbidity in all of the samples that were incubated with trypsin (regardless of the concentration used), we plotted the results as the difference of turbidity between samples that were incubated with PKME or with PKME/Ca^{2+}, normalized to the signal in the absence of Ca^{2+} at each concentration of inhibitor.

RESULTS

CG–CG Fusion Observed with DIC and Fluorescence Optics. To define the compositional requirements for exocytosis, we asked if cortical granules will fuse together in response to Ca^{2+}. We purified cortical granules from sea urchin eggs and concentrated them by centrifugation, creating monolayers of granules. Because the granules are large (about 1 μm in diameter) and refractile, we were able to follow fusion visually by using DIC optics, which are highly sensitive to any spatial discontinuity of refractive index. We perfused these clumps of granules with a buffer containing Ca^{2+}. After perfusion we observed the disappearance of high-contrast edges between individual small granules and the progressive relaxation of membrane curvature until single, birefringent edges remained enclosing refractile cores (Fig. 1 *a–d*). Focusing through the former clump of spherical granules revealed pancake-like structures. These morphological changes appeared consistent with coalescence of granule contents and fusion of granule membranes. Spontaneously, the cores of these structures sometimes radially dispersed over a few video frames (Fig. 1 *d* and *e*). This is consistent with a bursting of the fused membrane, releasing its contents to the surrounding buffer. As noted previously (10, 11), we found that only 1% of single isolated granules lysed after perfusion with 174 μM Ca^{2+} (4 of 388 observations). Pairs of isolated vesicles rarely exhibited a Ca^{2+}-triggered morphological change (8%; 13 of 168 doublets observed), even when in proximity to but not contiguous with fusing granules (Fig. 1). In contrast, clumps of vesicles exhibited the aforementioned changes with high probability when exposed to buffers containing 174 μM Ca^{2+}. For example, 30% (11 of 37 observations) of isolated triplets appeared to fuse when perfused with buffers containing Ca^{2+}. When large clumps or sheets of cortical granules were exposed to buffers containing Ca^{2+}, this morphological change often began at a discrete point and then spread sequentially through adjoining granules (Fig. 1 *h–m*). During the formation of these structures, we always observed both merging of granule contents and coalescence of granule membrane; hereafter we refer to these structures as "fusosomes." We sometimes observed bulk flow of contents through lesions in the fusosomal membrane. In these instances we noticed intrafusosomal particles moving under what appeared to be the surface of a newly formed membrane, over distances much greater than the diameter of single granules, suggesting once again that the individual granule membranes had fused to form one large membrane surrounding the amalgam of granule contents (CG–CG fusion).

To determine whether the granule membranes do indeed fuse with each other and whether their lipids freely mix, we labeled individual granules with the fluorescent membrane probe Di-I. Within 1 min of Ca^{2+} perfusion, we observed a rapid transfer of dye from singly labeled granules to their abutting unlabeled neighbors (Fig. 1 *f* and *g*). This rapid dye transfer was accompanied by fusosome formation. Minutes after Ca^{2+} perfusion, we always observed many large circular, fluorescent fusosomes. Perfusion with buffers containing <1 μM Ca^{2+} did not cause fusion, as evidenced by DIC optics, nor did we observe rapid transfer of dye from granule to granule. Fluorescent lipid probes may also transfer by nonfusional mechanisms. Consistent with this, we observed a slower transfer of dye from granule to granule on the time scale of 1 hr in the absence of Ca^{2+}. Pretreatment of granules with either of two inhibitors of planar cortex exocytosis, NEM or trypsin, did not inhibit the slow transfer of dye. However, it did inhibit both the Ca^{2+}-dependent morphological changes observed with DIC optics (see below) and the rapid transfer of Di-I. While we never observed transfer of dye across the aqueous gaps between granules that we could resolve with the light microscope, we cannot rule out a mechanism that transfers dye across gaps that we could not resolve (<0.13 μm). Thus, the observed slow transfer of dye suggests that the distances between granule membranes were small, even after treatment with trypsin or NEM. Regardless of the cause of slow dye transfer, the extent and speed of dye transfer in these cases were trivial compared to the transfer observed in native granules upon the introduction of Ca^{2+}.

Content Dispersal Is a Distinct Step from Membrane Fusion. After triggering fusion, we found two forms of fusosomes formed from individual granules. In the first fusosomal form (Fig. 1*n*), we observed bumps on the fusosomal membrane that corresponded to the outlines of the individual granule contents. Other than a subtle morphological change that appeared as a melding of the granule contents, there was no apparent change of state of the granule contents. We believe that the contents of this form of fusosome are not dispersed because Brownian motion was not observed. In the second fusosomal form (Fig. 1*o*), we observed a smooth rim on the fusosomal membrane and rapid Brownian motion of particles inside, consistent with a major change in state of their contents. The second form was derived from the first, and this transition, when it occurred, was often accompanied by the explosive release of the granule contents into the surrounding buffer.

Ca^{2+} and Contact Are Required for Fusion. To determine the concentration of free Ca^{2+} necessary to trigger CG–CG fusion, to confirm our morphological observations of CG–CG fusion, and to facilitate and expedite fusion measurements, we developed a microtiter dish fusion assay based upon the change in light scattering that accompanies cortical granule exocytosis (2, 3, 5). When large clumps of cortical granules fuse, their contents disperse into a number of less compact structures. Light scattering increases as the square of the mass of each particle (16), and scattering is less for dispersed structures than for compact structures of the same mass (17). Thus, light scattering decreases as cortical granule contents disperse. At Ca^{2+} concentrations > 1 μM, we observed a decrease in light scattering of purified cortical granules in

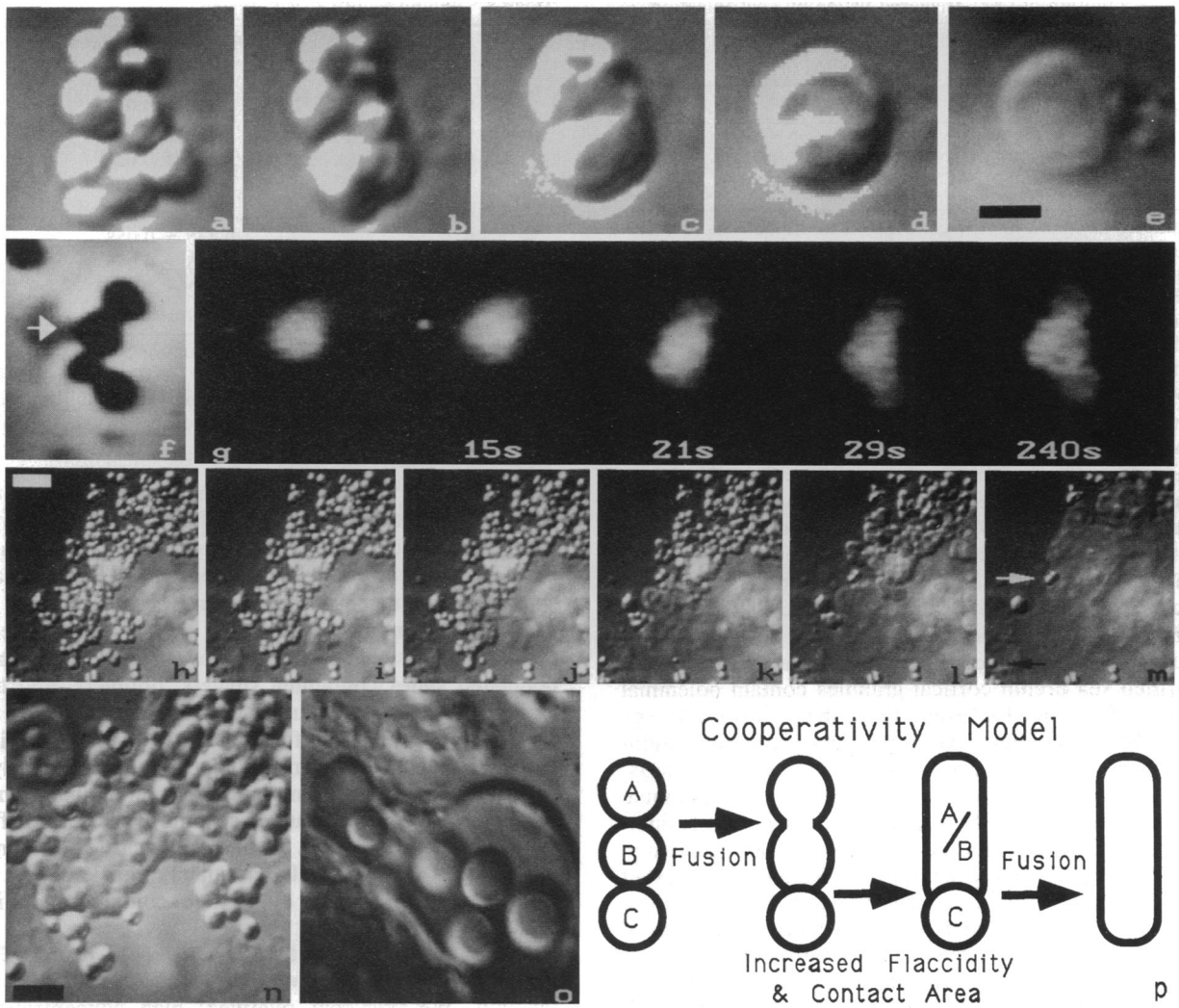

FIG. 1. (*a–e*) CG–CG fusion observed with DIC optics. A small clump of cortical granules can fuse with each other in response to a 174 μM Ca^{2+} trigger. Cortical granules were pelleted onto glass coverslips to form clumps and then perfused with Ca^{2+} to trigger vesicle–vesicle fusion. Fusosome formation without an apparent change of state in the granule contents is shown in *a–d*, and lysis of the fusosome is seen in *e*. (Bar in *e* = 1 μm.) (*f* and *g*) CG–PM fusion observed by fluorescent dye transfer. A single cortical granule, in a clump of four granules, was labeled with Di-I (arrow) and visualized with phase-contrast optics (*f*). The fluorescent image of this clump of granules prior to perfusion with 115 μM Ca^{2+} and at various times after perfusion is seen in *g*. Note that noncontacting granules (e.g., one in the upper left-hand corner) do not become fluorescent. (*h–m*) Time sequence of Ca^{2+}-triggered CG–CG fusion after perfusion with PKME containing 174 μM free Ca^{2+}. Time after initiation of Ca^{2+} perfusion: 4.98 s (*h*), 9.63 s (*i*), 11.20 s (*j*), 12.21 s (*k*), 14.54 s (*l*), and 25.32 s (*m*). The black and white arrows in *m* indicate examples of a single granule and doublet that have not lysed or fused in response to Ca^{2+} perfusion. (Bar in *h* = 5 μm.) (*n* and *o*) Two forms of fusosomes are observed after CG–CG fusion. After 174 μM Ca^{2+} perfusion, the first fusosome form (*n*), which does not show internal Brownian motion, appears as a flattened pancake composed of homogeneous contents within a containing membrane having an irregular outline. The second form (*o*) also looks like a pancake, but the containing membrane has a smooth outline, and the contents appear to have separated into dense, round inclusions within a clear liquid. These inclusions undergo Brownian motion within the surrounding membrane. (*p*) Diagram showing how the fusion of granule A and B can increase the probability of fusion between fusosome A/B and granule C, when the probability of fusion is a function of surface area of contact. (Bar in *n* = 5 μm.)

solution (Table 1). If we centrifuged the same granules to enhance CG–CG contact, we found a further decrease in light scattering at higher Ca^{2+} concentrations. When we first pelleted the granules and then added Ca^{2+}, we found the same decrease in light scattering (data not shown). Because centrifugation caused no change in light scattering at Ca^{2+} concentrations < 1 μM, the decrease in light scattering was not due to centrifugation alone (Table 1). When observing fusion in continuous sheets microscopically, we first observed fusion of all contiguous granules, later followed by leakage of contents out of the large fusosomes (Fig. 1 *h–m*). Thus, the decrease in light scattering is a result of fusion. The calcium concentration required to trigger half maximal scattering change ($\approx$30 μM) was similar to that reported for reconstituted cortical granule-to-PM fusion (CG–PM fusion) (10, 11, 18) and was higher than the Ca^{2+} sensitivity of exocytosis of intact planar cortices, 5 μM (Fig. 2).

Protein Modifiers Inhibit CG–CG. CG–PM fusion can be inhibited by trypsin treatment of isolated cortical fragments, suggesting that proteins sensitive to trypsin are involved in the cortical reaction (19). We found that trypsin treatment of granules, under conditions similar to that which inhibits exocytosis in cortices, prevented them from fusing with each other, despite the presence of $\approx$350 μM calcium (Table 2). Jackson and colleagues have also shown that the sulfhydryl-modifying reagent NEM can inhibit the cortical reaction (2, 19). NEM, at concentrations similar to those used by Jackson, inhibits Ca^{2+}-triggered CG–CG fusion in a dose-dependent manner, even at high concentrations of Ca^{2+} (Table 2).

Table 1. Promotion of Ca^{2+}-triggered fusion by centrifugation

Free Ca^{2+}, μM	Turbidity of cortical granules, mean OD_{405} ± SEM (n = 5)		
	In solution	Spun*	Background†
0.18	0.108 ± 0.001	0.109 ± 0.005	0.045 ± 0.005
42	0.084 ± 0.001	0.071 ± 0.004	0.040 ± 0.001
343	0.078 ± 0.001	0.054 ± 0.004	0.038 ± 0.003

*The samples in solution were centrifuged to the bottom of microtiter-dish wells to force CG–CG contact, and the turbidity was again measured. The turbidity of the centrifuged samples in the presence of both 42 and 343 μM Ca^{2+} was significantly different from that of the sample in solution as determined by a paired Student t test ($P < 0.011$ and 0.001, respectively).

†Background absorbance was measured by lysing remaining granules with water.

DISCUSSION

We find that isolated secretory granules fuse to each other in response to Ca^{2+}. Thus, they carry sufficient components to mediate Ca^{2+}-triggered fusion. Fusion was detected by transfer of fluorescent membrane marker and by observing the melding of granule contents using DIC optics. Fusion is enhanced by increasing the number of CG–CG contacts and is inhibited by trypsin and NEM.

Several lines of evidence argue against the possibility that our purified sea urchin cortical granules contain oolemmal contamination needed for fusion. First, the *S. purpuratus* egg surface (PM plus vitelline coat) binds strongly to polycation-coated surfaces (8). As opposed to *Lytechinus pictus* eggs, which stuck with less avidity and did detach during granule isolation, *S. purpuratus* PMs remained on the poly(D-lysine)-coated tissue culture flasks and were not visually detected in the final cortical granule preparation. (We have also tested purified *L. pictus* granules, and they fuse together in response to Ca^{2+}.) Second, to control for the possibility that clumps of granules contain small fragments of oolemma, granules were passed through mesh to break up clumps and then were passed through polylysine-treated glass wool to retain residual PM fragments. CG–CG fusion was still observed. Third,

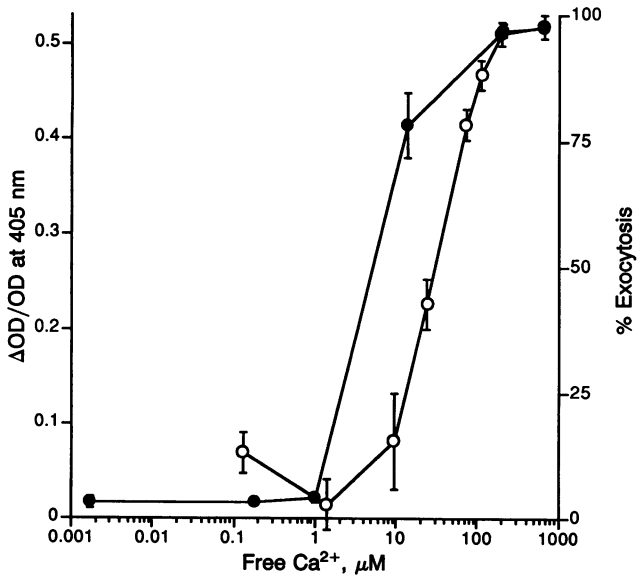

FIG. 2. Ca^{2+} sensitivity of CG–CG fusion. The extent of exocytosis in isolated planar cortices (●) and CG–CG fusion (○) was plotted as a function of Ca^{2+} concentration. To compare CG–CG fusion with % exocytosis in isolated planar cortices, we plot the data as the change of OD normalized to the initial OD. ΔOD/OD was calculated by the equation (OD_{405} before Ca^{2+} − OD_{405} after Ca^{2+})/(OD_{405} before Ca^{2+}). All points represent the mean ± SEM (n = 5).

Table 2. Inhibitors of CG–CG fusion

Inhibitor	ΔOD/OD at 405 nm*	n
Trypsin (mg/ml)†		
0	0.457 ± 0.039	5
0.05	0.514 ± 0.047	5
0.5	0.413 ± 0.021	5
1.0	0.018 ± 0.026	5
NEM (mM)		
0	0.435 ± 0.058	3
0.1	0.465 ± 0.068	3
1.0	0.092 ± 0.008	3
5.0	0.009 ± 0.046	3

*ΔOD/OD was calculated by the equation (OD_{405} with PKME −OD_{405} with PKME + 350 μM Ca^{2+})/(OD_{405} with PKME). All values are means ± SEM. Coincubation with 10 mM benzamidine or 1 mM phenylmethylsulfonyl fluoride prevented the inhibition caused by trypsin.

we saw stable fusosomes whose contents had not been dispersed resulting from CG–CG fusion. Fusion of granules with PM fragments, which exist as sheets of membrane (3), always results in explosive content release (5). Finally, if small PM fragments with adherent granules caused Ca^{2+}-triggered fusion, then we would see fusion in the same proportion for single granules as for clusters. When sparsely populated slides containing singlets, doublets, and triplets were perfused by Ca^{2+}, fusion was disproportionately higher for triplets (e.g., 1% lysis for singlets, 30% fusion for triplets). Indeed, isolated triplets and doublets that do fuse rarely lyse, suggesting that a small amount of PM contamination may be responsible for granule and fusosome lysis but not for the observed CG–CG fusion. In the large aggregates formed in our microtiter wells (≈10^8 granules), this 1% lysis would guarantee the dispersal of granule contents seen in our scattering assays.

The finding that both trypsin and NEM inhibit CG–CG fusion suggests that granule proteins mediate the fusion reaction. We find that relatively high concentrations of trypsin or NEM are required to inhibit CG–CG fusion. In addition, this inhibition is not linear with concentration. However, similar results were obtained for inhibition of intact CG–PM fusion (19). This suggests that the same sites are affected in both the intact and reconstituted preparations. Because trypsin is thought to be membrane impermeant, the trypsin-sensitive site is probably located on the outer surface of the granule. While it is possible that trypsin might inhibit fusion indirectly by perturbing membrane structure, gross structural changes seem unlikely because digested granules do not lyse in the absence of Ca^{2+}, even under the stress of centrifugation. The finding that, in reconstituted CG–PM systems, chymotrypsin treatment of egg PMs prevents native cortical granule binding suggests that proteins tether granules onto the surface of the PM (18). Nonetheless, it is unlikely that the inhibition of vesicle fusion by trypsin is solely due to preventing CG–CG adhesion because (*i*) trypsin treatment of cortical granules enhances their aggregation (18), (*ii*) proteolysis does not remove cortical granules from isolated cortices (19), (*iii*) trypsin treatment did not stop the slow transfer of Di-I from one granule in a clump to other granules, and (*iv*) treated granules did not fuse in the presence of Ca^{2+} even when they were pressed together by centrifugal force. While it is possible that proteolytic fragments of proteins that are not involved in vesicle fusion might occlude essential sites of the fusion mechanism, the simplest interpretation of our data is that CG–CG fusion is mediated by a trypsin-sensitive protein that resides on cortical granules. Because the Ca^{2+}-triggered transfer of cortical granule membrane-bound dye (Di-I) is inhibited by both trypsin and NEM, the affected

components must be involved in the initial fusion of the membranes rather than some later stage.

Physiological Significance. Is CG–PM fusion in the native sea urchin cortex mediated by the same components as the CG–CG fusion we have observed? A difference between the two systems is the concentration of Ca^{2+} required for fusion. The Ca^{2+} required for half-maximal fusion [Ca^{2+}_{50}] is 2.5–4.5 μM in eggs (20), $\approx$5 μM in our intact cortices, and 30 μM in CG–CG fusion. However, the [Ca^{2+}_{50}] in reconstituted CG–PM fusion is at least as high as 25 μM (10, 11, 18). Thus, the shift of Ca^{2+} sensitivity is correlated with reconstitution itself. One explanation for the shift of Ca^{2+} sensitivities upon reconstitution is suboptimal contact between the reacting membranes, a variable that we have shown can effect the extent of fusion (Table 1). Another explanation could be a loss of factors during granule isolation (21). Alternatively, a higher concentration of fusion proteins in the native attachment site on the PM may yield a lower Ca^{2+} requirement. Whatever the mechanism, the fusion seen here is significantly different from that seen with lipid vesicles, where both millimolar concentrations of Ca^{2+} and exogenous osmotic stresses are required (22).

A similarity between CG–CG fusion and exocytosis *in situ* is seen in fast-frozen freeze-fracture replicas of the intact cortex, where the same fusion site morphology is found between PM and the cortical granule as well as between adjacent cortical granules (5).

Cooperativity. We have found that large clumps of granules fuse in response to Ca^{2+}, while pairs of granules rarely fuse (Fig. 1). In addition, single isolated granules readily fuse with egg PM (data not shown, confirming refs. 10 and 11). Whereas a high concentration of fusion sites on the PM could explain this last observation, we do not know if the PM has fusion components as do the cortical granules, nor would fusion sites on the PM explain the cooperativity observed in CG–CG fusion, where no PM is present. Whalley and Whitaker (11) have suggested that a lipid-soluble substance might be responsible for this behavior. Since we have observed speeds of CG–CG fusion consistent with a diffusion constant of 4×10^{-6} cm^2/s (measured from the site of initiation to the edge of the clump), and since the diffusion constant of a lipid in a membrane is typically 10^{-8} cm^2/s, this explanation seems unlikely. Although this speed is consistent with a water-soluble second messenger such as Ca^{2+}, we think this unlikely because a pair of granules, surrounded by but not contiguous with a large clump of granules, fails to fuse despite fusion activity all around it (see black arrow in Fig. 1).

Alternatively, a physical change may occur at the granular membrane–membrane contact site when a neighboring granule fuses, such as an increase in CG–CG contact area (see Fig. 1*p*). In a clump with many granules, there would be a high probability that at least one CG–CG contact was permissive for fusion. If those granules fused, their membrane flaccidity would increase. The increase in flaccidity would now allow a greater area of contact between that fusosome and adjacent granules. If the probability of fusion were a function of the surface area of contact, cooperativity would be observed.

This model also suggests explanations for both compound exocytosis, where there is a striking and rapid change in the fusibility of granular membranes upon their fusion to the PM (23–25) and for individual cortical granule fusion with the egg PM. A large contact area is predicted between granules and PM since PM is flaccid, so granules should readily fuse. Once fused to the PM, the increased flaccidity of the granule membrane would allow greater contact with neighboring granules and, hence, increased fusibility.

Model Systems for Fusion. Vesicle–vesicle fusion has been observed between isolated secretory vesicles from several cell types (26), but fusion is inefficient. For example, in neurosecretosomes <5% vesicle–vesicle fusion was observed at 100 μM free Ca^{2+} (27), and in zymogen granules, 2–3% fluorescence dequenching due to fusion was observed (M. Edwardson, personal communication; see also ref. 28).

Only two secretory systems yield efficient CG–CG fusion *in vitro*: sea urchin cortical granules as reported here and purified chromaffin granules (29). However, there are significant differences between these two systems: (*i*) chromaffin granules will only fuse when a cytoplasmic protein, such as synexin, is present; (*ii*) for chromaffin granules to fuse, there is an absolute requirement for reagents such as arachidonic or oleic acid (30); and (*iii*) unlike the process in sea urchin, fusosomes formed from chromaffin granules always appear as if their contents have been dispersed. Thus, the pathways leading to fusion may be different in these two systems.

In summary, we have shown by reconstitution that cortical granules carry components, at least a part of which are proteinaceous, that enable their fusion with biological membranes in response to a Ca^{2+} trigger. CG–CG fusion does not require any cytoplasmic proteins or organic factors. We present a simple assay for detecting the fusion of many samples at one time, which should facilitate the characterization of the fusion reaction.

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