Proc. Natl. Acad. Sci. USA
Vol. 74, No. 6, pp. 2417–2421, June 1977
Cell Biology

Membrane ultrastructural changes during calcium phosphate-induced fusion of human erythrocyte ghosts

(intramembrane particle rearrangement/phospholipid exposure to enzyme/model for membrane fusion)

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Communicated by Harland G. Wood, February 7, 1977

ABSTRACT **Nascent calcium phosphate promotes the agglutination and fusion of human erythrocyte ghosts. Membrane phospholipids of erythrocyte ghosts treated with Ca^{2+} and phosphate ions become exposed to attack by phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) (*Bacillus cereus*). Freeze-fracture pictures of fused erythrocyte ghosts show the presence of regions deficient in intramembrane particles in the protoplasmic face which we believe to be regions of fusion. Discontinuous regions of the protoplasmic and exoplasmic faces are observed, which are apparently intermediate stages in the process of fusion. Thin-section electron micrographs reveal deposits of calcium phosphate in areas of contact and fusion of ghosts. Ca^{2+} in the presence of *N*-[tris(hydroxymethyl)methyl]glycine (Tricine) buffer causes the formation of blebs in the membrane but does not cause changes in the intramembrane particle pattern or induce fusion. It is suggested that nascent calcium phosphate acts by forming protein-free regions of phospholipid bilayer which can fuse readily.**

Membrane fusion is a widely observed phenomenon which is still not well understood in spite of extensive research (1). Fusion systems can be divided into three main groups: (*a*) "natural," nonviral fusion systems which include secretion of enzymes (2), collagen (3), and hormones (4), fusion of differentiating myoblasts (5), pinocytosis (6), formation of secondary lysosomes (7), and conjugation of protozoa (8); (*b*) virus-induced fusion which, in addition to occurring naturally (9), has been exploited in various laboratories for studying the mechanism of membrane fusion (10, 11) as well as for somatic cell hybridization (12); (*c*) chemically induced fusion, which has been obtained with reagents that increase membrane mobility (13, 14).

A common denominator of almost all fusion systems is a requirement for Ca^{2+}. In the absence of Ca^{2+} fusion does not usually take place. For example, Sendai virus in the absence of Ca^{2+} causes lysis of cells rather than fusion (15). In certain systems Ca^{2+} can cause fusion either alone (16, 17) or with the aid of high pH (18) or calcium ionophore A-23187 (19, 20). Fusion of phosphatidylserine-rich vesicles with the aid of Ca^{2+} has been used as a model system to explain the mechanism of membrane fusion (17). It has been suggested that Ca^{2+} forms solid aggregates with phosphatidylserine in phospholipid bilayers (21). Segregation of phosphatidylserine might promote the melting of the other phospholipids in the bilayer and thus cause fusion. Because biological membranes are more complex than lipid vesicles and contain proteins, glycoproteins, and glycolipids in addition to simple lipids, the model proposed for liposomes may be too simple to explain the fusion of natural membranes.

Recently we have shown that nascent calcium phosphate is a powerful fusion agent for human erythrocyte ghosts (22). This system is simpler than other systems for studying fusion because the entire process requires only a combination of Ca^{2+} and phosphate ions. Erythrocyte ghosts, which are natural membrane vesicles of extensively studied chemical composition, are well suited for studies on the mechanism of fusion.

Freeze-etching studies in this and other laboratories showed that presumptive fusion areas are depleted of intermembrane particles (19, 22–25). In the present investigation we have studied in more detail the Ca^{2+} and phosphate ion-induced fusion of erythrocyte ghosts in an attempt to understand the essential steps of fusion. On the basis of electron microscope observations on ghosts fused with Ca^{2+} and phosphate, we have attempted to develop a model of the various steps of fusion of natural membranes.

Abbreviation: Tricine, *N*-[tris(hydroxymethyl)methyl]glycine.

MATERIALS AND METHODS

Preparation and Fusion of Human Erythrocyte Ghosts. Ghosts were prepared and fused as described before (22).

Phospholipase C Treatment of Fused Human Erythrocyte Ghosts. Ghosts were washed and suspended either in isotonic medium (120 mM KCl, 30 mM NaCl) buffered with 10 mM sodium phosphate, pH 7.4, or in the same isotonic medium buffered with 20 mM tricine, pH 7.4. Fusion was initiated by adding $CaCl_2$ at a final concentration of 2 mM to 6 ml of 5% (vol/vol) ghost suspension. After 25 min incubation at 37°, each system was divided among two tubes and centrifuged for 10 min at 20,000 × *g*. The ghosts in each tube were suspended in 5 ml of solution containing 5.3 mM NaCl, 135 mM KCl, 0.8 mM $MgSO_4$ buffered with 20 mM *N*-[tris(hydroxymethyl) methyl]glycine (Tricine), pH 7.8 (solution K). Phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) (*Bacillus cereus*) was added at a final concentration of 20 μg/ml and the ghosts were shaken in an ice bath for 60 min. Phospholipase C digestion was performed in the cold in order to avoid further effects of calcium phosphate during incubation at 37°. The ghosts were then washed twice in solution K (20,000 × *g* for 15 min) and finally the supernatant was discarded.

Determination of Phospholipid Hydrolysis by Phospholipase C. Membrane phospholipids were extracted according to Burger *et al.* (26). The amount of phosphate in membranes was estimated according to Bartlett (27).

Preparation of Samples for Electron Microscopy. Samples were prepared and sectioned as described previously (10).

Freeze-Etching. Freeze-fractured cells were prepared essentially as described before (22) with the following modifications. Erythrocyte ghosts were fixed at 37° immediately after fusion by addition of glutaraldehyde (Ladd Research Industries) to a final concentration of 1% (vol/vol) and centrifuged at 12,000 × *g* for 5 min. All subsequent centrifugations were also at 12,000 × *g* for 5 min.

RESULTS

Role of Nascent Calcium Phosphate in Agglutination and Fusion of Human Erythrocyte Ghosts. Human erythrocyte

Table 1. Effect of Ca^{2+} and ionophore A-23187 in presence and absence of poly(L-lysine) on fusion of human erythrocyte ghosts in Tricine or phosphate buffer

Buffer	Addition	Agglutination	Fusion
Tricine	None	–	–
	Ionophore	–	–
	Ionophore + poly(L-lysine)	+++	–
Phosphate	None	+++	+++
	Ionophore	+++	+++
	Ionophore + poly(L-lysine)	+++	+++

Preparation of human erythrocyte ghosts and their fusion were as described in *Materials and Methods*. $CaCl_2$ (2 mM) was added to each system just before transferring to 37°. In systems that contain ionophore and poly(L-lysine), ghosts were preincubated for 5 min at 37° with ionophore A-23187 (2 μg/ml) and $CaCl_2$ and then poly(L-lysine) (10 μg/ml, average chain length = 133 residues) was added and incubation at 37° was continued for 20 min. Agglutination and fusion were estimated by phase contrast microscopy. Agglutination: +++, clumps of 50 to 100 cells. Fusion: +++, large polyghosts derived from 10 to 20 cells and 60–80% of ghosts fused.

ghosts fused when Ca^{2+}, final concentration 2 mM, was added at 37° to a suspension of ghosts in isotonic KCl containing 10 mM phosphate buffer. Both Ca^{2+} and phosphate ions were essential for fusion. Table 1 shows that Ca^{2+} and the ionophore A-23187 did not promote fusion in Tricine buffer even when the ghosts were agglutinated with poly(L-lysine). In phosphate buffer fusion was observed both in presence and in absence of ionophore, with or without poly(L-lysine).

Several steps in the fusion process could be discerned. After 5–10 min there was massive agglutination (Fig. 1*A*) and after longer incubation (15–25 min) the ghosts fused (Fig. 1*B*). On still longer incubation the ghosts lysed and sometimes disintegrated. Fig. 2 shows three stages in the fusion of human erythrocyte ghost membranes. First, agglutination with deposits of what we presume to be crystalline calcium phosphate in contact areas of adjacent cells. In the next step the area of contact becomes extended and disrupted, forming a line of vesicles ("seam") in which membranes are very dense, probably because of adhering calcium phosphate. Finally, the contiguous membranes of adhering cells melt, leaving traces of membrane in a line marking the region of fusion. At this stage of fusion crystalline deposits are no longer visible inside the cells.

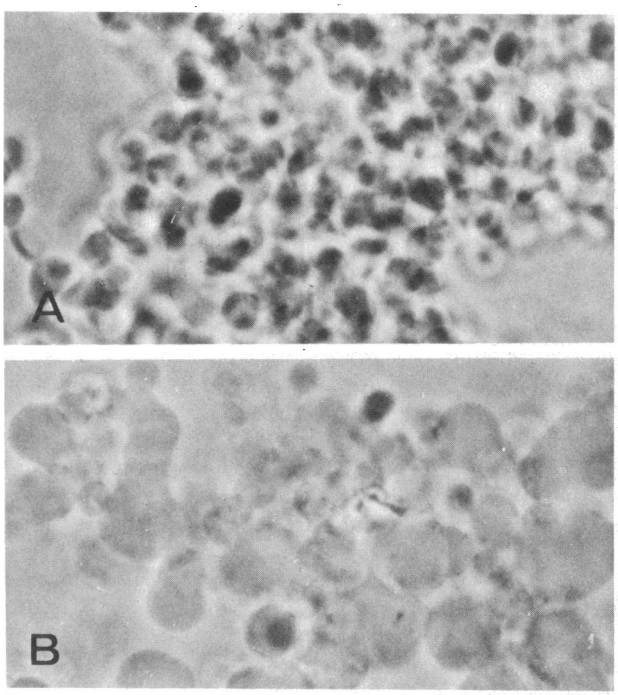

FIG. 1. Phase-contrast micrographs of agglutination and fusion of human erythrocyte ghosts. (×460.) (*A*) Ghosts agglutinated after 10 min incubation at 37° in a medium containing Ca^{2+} and phosphate. (*B*) Ghosts fused after an additional 15 min incubation at 37°.

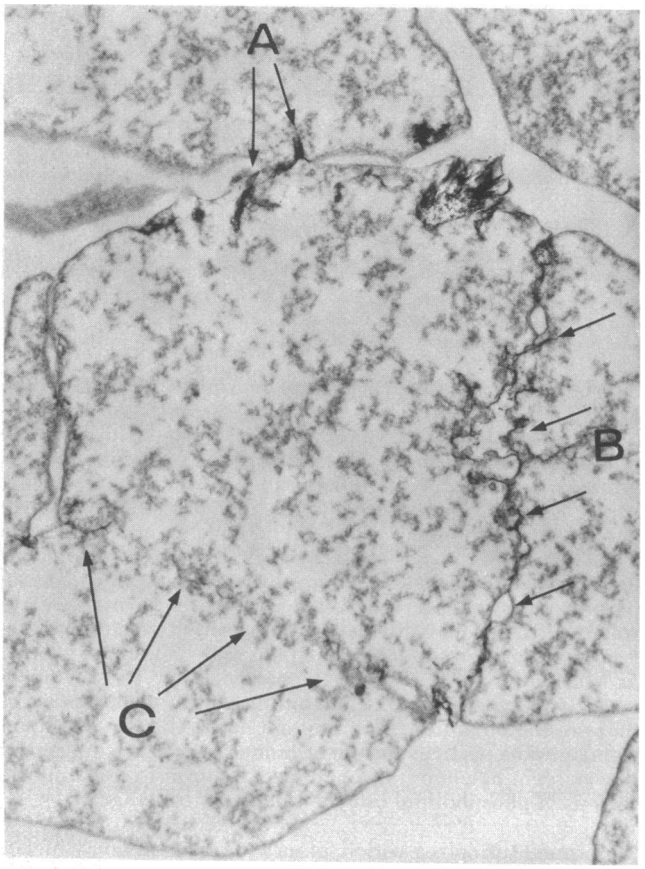

FIG. 2. Thin-section electron micrograph of human erythrocyte ghosts in the process of fusion. Arrows A: calcium phosphate, some of which is crystalline, at periphery of agglutinated ghost. Arrows B: ghosts in the process of fusion, showing line of vesicles and dense regions of membrane, possibly due to calcium phosphate deposits. Arrows C: traces of membrane marking former boundaries of fused cells. (×16,000.)

Changes in the Pattern of Intramembrane Particles during Fusion. Fig. 3*A* is a freeze-fracture picture of the protoplasmic face (PF) (28) of erythrocyte ghosts in the process of fusion. The figure shows a local discontinuity in the protoplasmic fracture face, which we interpret to be an area of close contact between two ghosts, merging with a continuous, presumably fused, membrane. It should be noted that the membrane surrounding the contact and presumed fusion regions is depleted of intramembrane particles. Fig. 3 *B* and *C* shows fusion "seams" in the exoplasmic fracture face (EF) (28) of erythrocyte ghosts in the process of fusion. Fig. 3*B* shows an early stage of fusion with extensive contact areas and some membrane bridges. Fig. 3*C* shows a more advanced stage of fusion with extensive merging of membranes interrupted by short regions of contact of what we presume to be two ghosts.

Fig. 4 *A* and *B* shows blebs on the protoplasmic fracture face

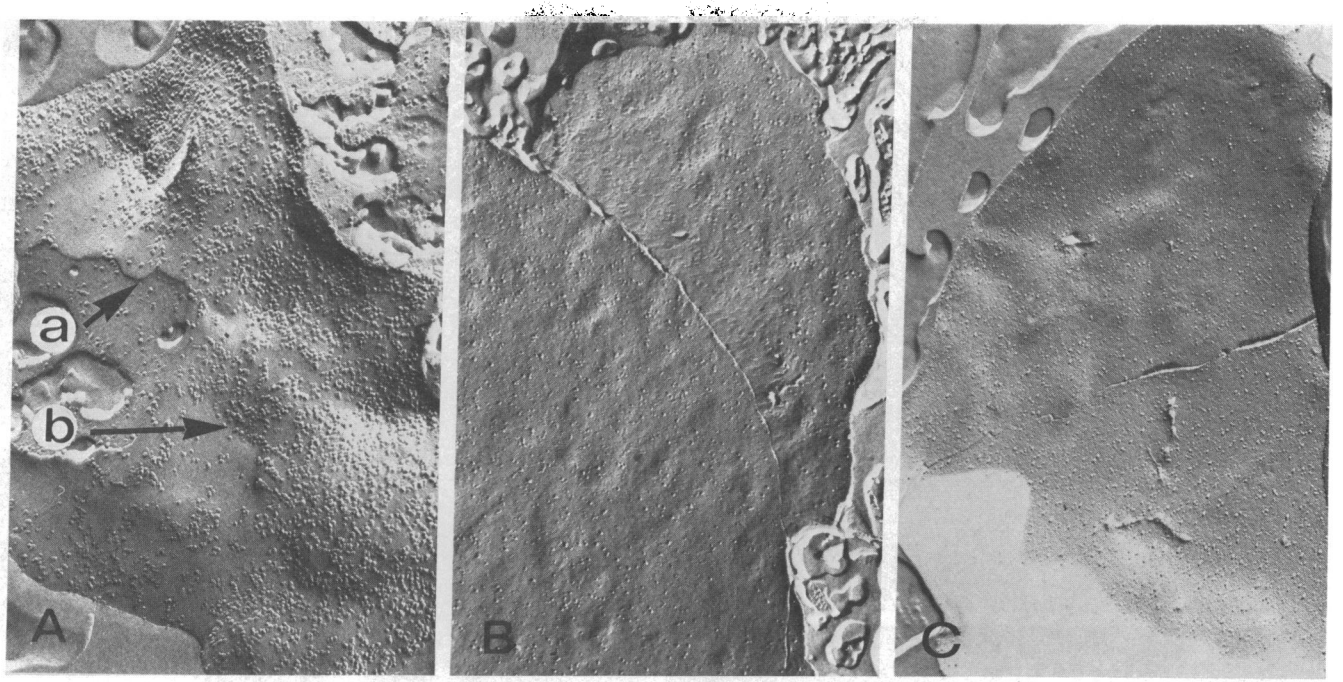

FIG. 3. Freeze-fracture pictures of human erythrocyte ghosts in the process of fusion. (*A*) Protoplasmic fracture face (PF) of fusing ghosts. Arrow a: a discontinuity ("seam") which is probably a region of contact between fusing cells. Arrow b: presumed region of fusion. Note sparseness of intramembrane particles in the area adjacent to the "seam." (×48,000.) (*B* and *C*) Exoplasmic fracture face (EF) of ghosts in the process of fusion. Note "seams" in contact regions of ghosts. In *C* fusion is more advanced and the "seam" is interrupted by fused membrane areas. (*B*, ×32,000; *C*, ×41,500.)

(PF) of erythrocyte ghosts incubated with nascent calcium phosphate. The blebs are devoid of intramembrane particles and are probably lipid vesicles containing little membrane protein. In both Fig. 4*A* and 4*B* blebs emerge from areas with a low density of intramembrane particles. Blebbing is also observed in the presence of Ca^{2+} under conditions that do not promote fusion, such as Ca^{2+} in Tricine buffer (not shown).

In freeze-fracture fields of ghosts treated with nascent calcium phosphate there are numerous small vesicles (0.1–1 μm in diameter) (Fig. 5). These are presumably blebs that have been pinched off and released into the medium. The vesicles are smooth with rare membrane particles. Fig. 5 shows a clump of four such vesicles, two of which are obviously in the process of fusion.

Calcium Phosphate-Induced Exposure of Membrane Phospholipids. Table 2 shows the effect of phospholipase C on phospholipids of erythrocyte ghosts treated with Ca^{2+} or phosphate buffer separately or together. When Ca^{2+} and phosphate buffer are added simultaneously under fusion conditions, extensive hydrolysis of phospholipids is observed, whereas when Ca^{2+} and phosphate are present separately there is only a slight release of phosphate from phospholipids. In the presence of Ca^{2+} and phosphate there is appreciable phospholipid release without phospholipase, which might be due to the pinching-off of blebs.

DISCUSSION

The electron micrographs showing deposits presumed to be calcium phosphate in contact areas between cells (Fig. 2) support our previous suggestion that nascent calcium phosphate acts as an agglutinating agent (22). Previous experiments also indicated that in addition to nascent calcium phosphate, intracellular Ca^{2+} is required for fusion (22). This might indicate that calcium phosphate acts simply as an agglutinating agent. However, experiments in which ghosts were incubated with free Ca^{2+}, calcium ionophore (A-23187), and an agglutinating agent (polylysine) did not result in fusion (Table 1). It seems, therefore, that in addition to causing agglutination, calcium phosphate plays an active role in fusion. Experiments in Lucy's laboratory (20) and in ours (Table 1 and unpublished experiments) showed that introduction of Ca^{2+} with ionophore A-23187 into human erythrocytes or their ghosts caused neither fusion nor change in distribution of intramembrane particles, probably because their intramembrane particles are immobile (29). In human erythrocyte ghosts fusion occurs and smooth areas are formed only when Ca^{2+} and phosphate ions are present together. Fusion is directly correlated with the appearance of smooth areas devoid of intramembrane particles in both chicken erythrocytes (20) and human erythrocyte ghosts.

The following model could explain the effect of calcium phosphate on human erythrocyte ghosts. When Ca^{2+} and phosphate buffer are mixed, a precipitate of calcium phosphate is formed, which causes the agglutination of the cells (Fig. 6*A*). Calcium and phosphate enter the cell, forming local precipitates that partially extract spectrin from the membranes. Under these conditions free intracellular Ca^{2+} or calcium phosphate, or both, cause the formation of smooth areas free of intramembrane particles (Fig. 6*B*). Elgsaeter *et al.* showed that partial depletion of spectrin from the membrane is a prerequisite for the aggregation of intramembrane particles by intracellular Ca^{2+} (29). Smooth areas might still contain membrane proteins that do not appear as intramembrane particles (30). Calcium phosphate, which is a protein adsorbing agent (31), might extract or concentrate these proteins from the membrane, thus forming a protein-free phospholipid bilayer (Fig. 6*B*). Then, under the influence of intracellular Ca^{2+}, fusion occurs in the region of the phospholipid bilayer. According to Lucy's model (32), micelle formation leads to fusion of the phospholipid bilayers (Fig. 6*C*). In the region of fusion, cytoplasmic bridges and membrane vesicles are formed. In Fig. 6*D* fusion is com-

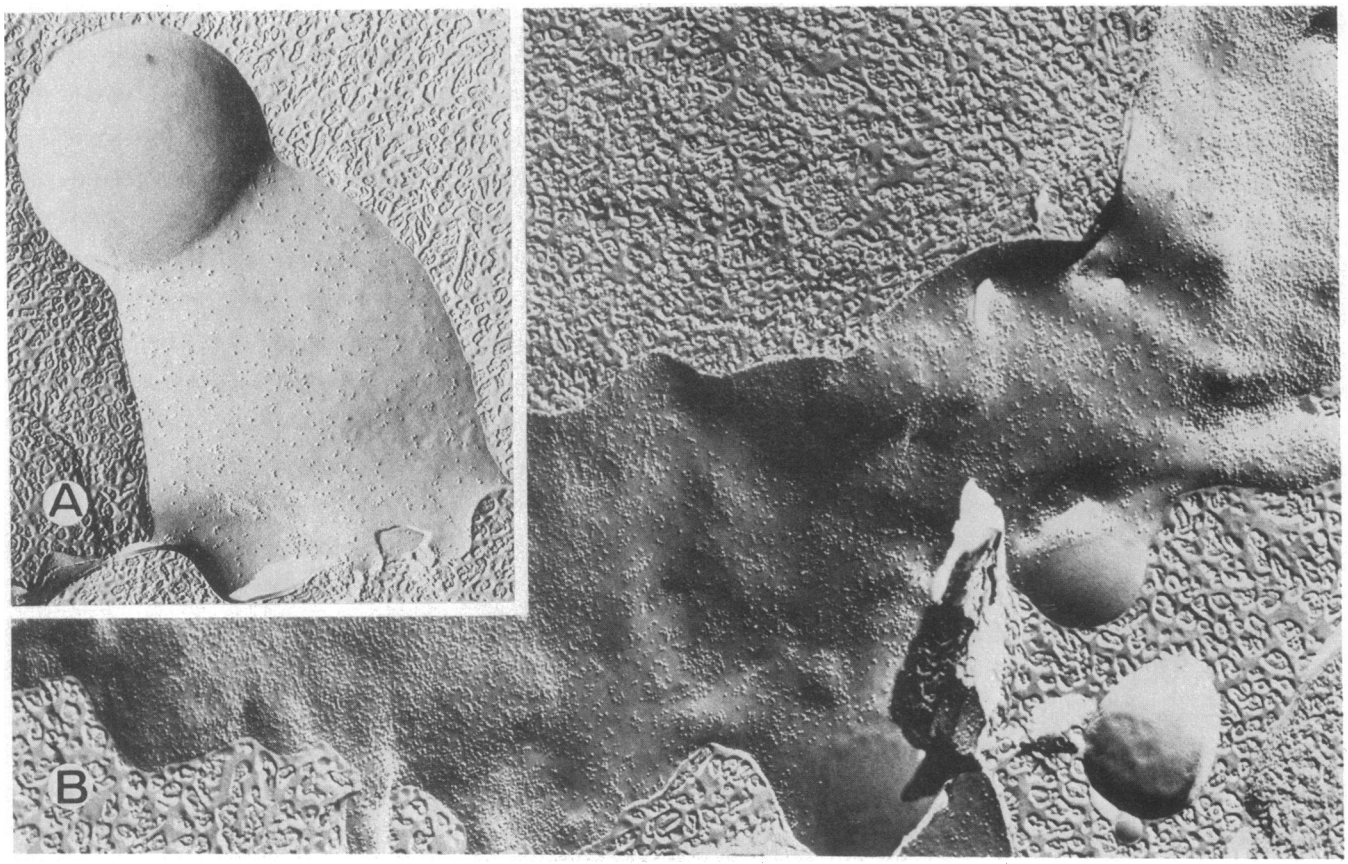

FIG. 4. Protoplasmic fracture face (PF) of membranes of fused ghosts in the process of blebbing. Blebs emerge from areas with a low density of intramembrane particles. (*A*, ×34,650; *B*, ×39,600.)

pleted and the cytoplasm of fused cells merges. Residues of membranes (liposomes and/or micelles) and membrane vesicles are discernible in the fused cell. In the final step (not shown in Fig. 6) fused cells become rounded and intramembrane particles become redistributed evenly on the membranes.

The following observations support the proposed model. Freeze-fracture pictures of the protoplasmic face (PF) show that areas free from intramembrane particles appear in fusing cells. Smooth areas formed in the presence of Ca^{2+} and phosphate are probably areas of exposed phospholipids, because this treatment exposed phospholipids to hydrolysis by phospholipase C (Table 1).

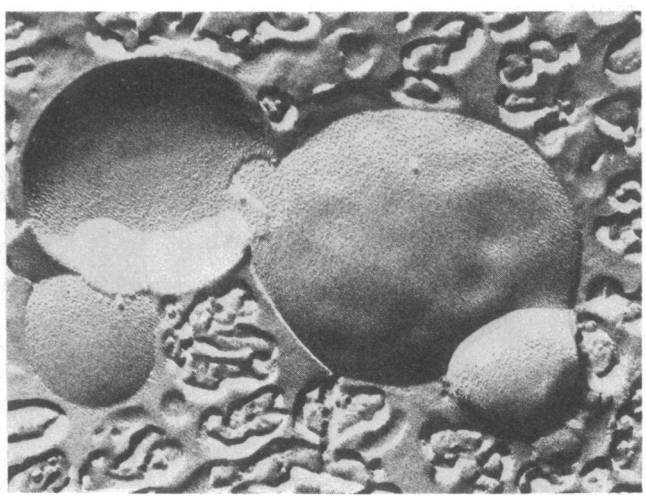

FIG. 5. Freeze-fracture of a clump of vesicles in the medium of fusing ghosts. The vesicles are presumably blebs that have been pinched off ghosts and released into the medium. Two of them are in the process of fusion. (×96,000.)

Lucy's hypothesis (20), as well as our observations (22), favors the view that fusion occurs in smooth membrane areas depleted of intramembrane particles. However, the freeze-etching technique does not distinguish between smooth membranes that contain integral proteins and pure lipid bilayers. It seems conceivable that the smooth areas undergoing fusion are pure phospholipid bilayers free of integral proteins.

In other membrane fusion systems, such as polymixin-induced secretion of histamine by mast cells (24), or virus-induced fusion of human and chicken erythrocytes (33, 34), presumptive fusion areas were reported to be depleted of intramembrane particles. It is tempting to suggest that in these systems the membranes that fuse are also pure lipid bilayers.

It is interesting that nascent calcium phosphate promotes the

Table 2. The effect of calcium phosphate on the degree of phospholipid hydrolysis by phospholipase C (*B. cereus*)

Buffer	$CaCl_2$	Phospholipase C treatment	Phospholipid release, %	Fusion
Tricine	−	−	0	−
	−	+	0	−
	+	−	2.7	−
	+	+	8.1	−
Phosphate	−	−	0	−
	−	+	8.9	−
	+	−	19.1	+++
	+	+	66.6	+++

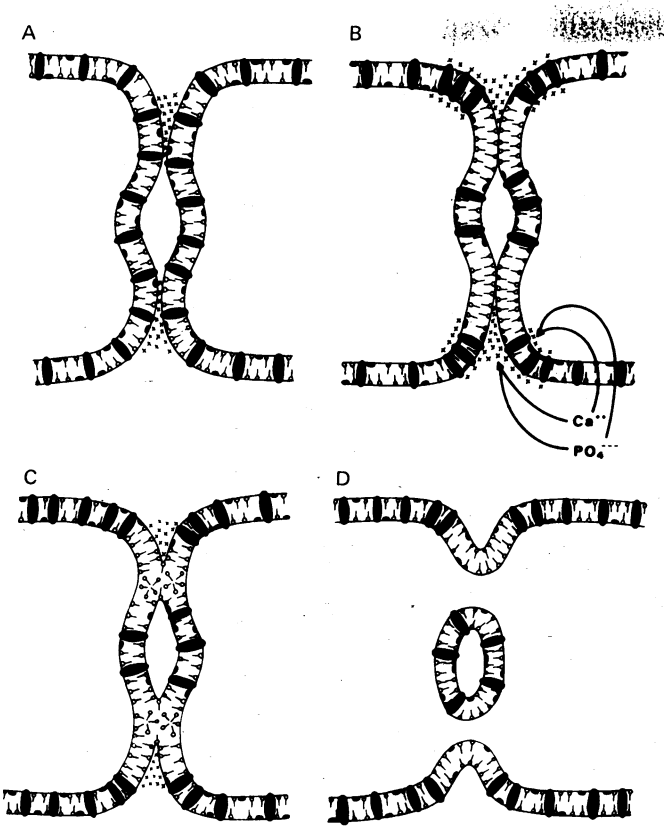

FIG. 6. Model for calcium phosphate-induced fusion of membranes. (*A*) Agglutination by calcium phosphate. Membrane proteins are randomly distributed. (*B*) Movement of membrane proteins and formation of phospholipid bilayer. Ca^{2+} and phosphate ions reach the inner side of the membrane and form a precipitate. Membrane proteins are aggregated by calcium phosphate. (*C*) Fusion of phospholipid bilayers. (*D*) Fusion completed with vesicle formation. ●, intramembrane protein; ▬, integral protein; ♀, phospholipid molecule; ×, calcium phosphate precipitate.

fusion of bacterial protoplasts (35) as well as the uptake of DNA by mammalian tissue culture cells (36). Perhaps both these processes involve a mechanism similar to the fusion of ghosts.

We thank Mrs. Y. Reichler for her skilled help in electron microscopy and freeze etching. This work was supported by a Grant Az.11 2454 from the Stiftung Volkswagenwerk to A.L.

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