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INHIBITION OF MEMBRANE FUSION BY SUPPRESSION OF LATERAL MOVEMENT OF MEMBRANE PROTEINS

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Summary

Chicken erythrocytes were fused either by *Sendai* virus or by the combination of Ca^{2+} and ionophore A23187.

Intramenbrane particles and external anionic sites of cells undergoing fusion were found to acquire the ability to undergo a process of cold-induced clustering (thermotropic separation).

Cationized ferritin (200 μg/ml 5% (v/v) cell suspension) inhibited both the fusion process and the thermotropic separation of intramembrane particles and external anionic sites. The correlation between the mobility of membrane proteins and the fusion process is discussed. It is suggested that an increase in the lateral mobility of membrane proteins is a prerequisite for initiation of membrane fusion.

Introduction

Recent results from various laboratories strongly suggest that membrane fusion occurs by intermixing of exposed phospholipids of adjacent cells [1—5]. However, the phospholipids in the intact membrane are masked and unavailable to external reagents [6,7]. It is therefore conceivable that phospholipids of one cell are unable to come into close contact with those of another cell, unless the proteins of both membranes are temporarily removed from the contact area.

A useful tool for studying the localization and distribution of membrane proteins with regard to the membrane phospholipids is the freeze-etching technique [4,8—10]. It has been shown that partial clustering of intramembrane particles, which leads to the formation of smooth areas in freeze-fractured membranes, results in the exposure of the masked phospholipids to attack by various phospholipases or trinitrobenzene sulfonic acid [6,7]. In several systems, the membrane fusion process is accompanied by a change in

the distribution of the intramembrane particles, as has been shown during secretion of histamine from mast cells [3], mating of *Chlamydomonas* [11] or during encystment of *Phytophtora palmivora* zoospores [4]. Thus, clustering of intramembrane particles, exposure of membrane phospholipids, and induction of membrane fusion, seem to be correlated. However, the possibility that the above-described correlation is only coincidental cannot be ruled out as yet. In order to clarify this question, it seems of extreme importance to study whether immobilization of membrane proteins would inhibit the membrane fusion process.

In the last few years we have studied the mechanism of membrane fusion by using human and chicken erythrocytes as a model system [5,12,13]. In these systems, no change in the pattern of intramembrane particles was observed during induction of membrane fusion. However, recently we have shown that the intramembrane particles of chicken erythrocytes did undergo a process of aggregation, when cells incubated with the fusogenic agents Ca^{2+} and the ionophore A23187, or with *Sendai* virus, were transferred from 37 to 4°C [13,14]. Such cold-induced clustering of intramembrane particles (thermotropic separation) may explain earlier reports by Bachi and Howe [15] on changes in intramembrane particles distribution during virus-induced fusion of human erythrocytes.

Cationized ferritin, in combination with native ferritin, was shown to inhibit thermotropic separation of intramembrane particles in mitochondria [16]. In the present work we have studied the effect of cationized ferritin on both virus- and Ca^{2+}-induced membrane fusion, and on thermotropic separation of intramembrane particles in chicken erythrocytes.

As the external surface of the cell membrane should play an active role in the first stages of the fusion process, it was also important to evaluate to what extent the cold-induced clustering of intramembrane particles was correlated to the movement of external groups. This was done by using cationized ferritin as a label of the cell's external anionic sites [10,17]. The fate of the ferritin label was followed at 37°C, where there is no clustering of intramembrane particles, and at 4°C, where clustering occurred [13]. Both, the thin sectioning technique and freeze-fracturing combined with deep-etching, were used.

Materials and Methods

Medium. The medium used throughout the present work (solution K) contained 135 mM KCl, 5.4 mM NaCl, and 0.8 mM $MgCl_2$ in 20 mM tricine-NaOH (pH 7.4).

Cells. Blood was collected from the necks of decapitated chickens into an Erlenmeyer flask containing heparin (100 units/ml). The blood was kept in the cold and used within 3 days. Before the experiment, cells were washed (three times) in solution K and the buffy coat was carefully removed. The final sediment was suspended in solution K to give 10 or 40% (v/v) concentration.

Virus. Sendai virus was propagated in the allantoic cavity of 10-day chicken embryoss, purified, and its hemagglutination determined, as previously described [12].

ATP depletion. ATP was depleted from chicken erythrocytes essentially as described before [7]. Briefly, washed erythrocytes (2.5%, v/v) were incubated for 8—12 h at 37°C with KCN (1 mM) and NaF (10 mM). At the end of the incubation period, the cells were washed three times in solution K and resuspended to the desired concentration.

Fusion of ATP-depleted chicken erythrocytes by the aid of Ca^{2+} and ionophore. Ca^{2+} was introduced into the chicken erythrocytes with the aid of the ionophore A23187 (obtained as a generous gift from Eli Lilly and Co., Indianapolis, Ind., and dissolved in ethanol, 0.3—3 mg/ml) as described in a previous paper [13]. For details, see legends to figures. Between 10 and 30 μl ionophore solution were added to each ml of cell suspension, and this was followed by the addition of the indicated amount of $CaCl_2$. Addition of $CaCl_2$ in the presence of an ionophore resulted in rounding and agglutination of cells, the extent of which depended on the $CaCl_2$ concentration [13]. Up to 60% of ATP-depleted chicken erythrocytes could be fused within 60—90 min of incubation at 37°C, while negligible fusion was observed in fresh cells under these conditions.

Coating of cells with cationized ferritin. Chicken erythrocytes suspended in solution K (5 or 40%, v/v) were incubated at 37°C and then cationaized ferritin (Miles-Yeda, Rehovot, Israel) was added (220 μg/1 ml suspension) either at 37°C or at 4°C. To ensure tight binding of cationized ferritin, the cells were further incubated in the presence of the particles for 10 min at the indicated temperature. Under the conditions described, the addition of cationized ferritin resulted in strong and lasting agglutination of cells.

Interaction of Sendai virus with chicken erythrocytes. Sendai virus (2000 HAU/ml of cell suspension) was added to cells (2.5%, v/v) which were incubated in the presence of $CaCl_2$ (20 mM) at 37°C, as described elsewhere [25]. Addition of the virus resulted in immediate agglutation which was followed by full-scale fusion within 30 min of incubation [25].

Measurement of Ca^{2+} uptake. Ca^{2+} accumulation in chicken erythrocytes was measured as described previously [13]. Briefly, cells were preincubated with an ionophore, as described above. $^{45}Ca^{2+}$ (carrier free, 2 mCi/ml, New England Nuclear, Boston, Mass.) was added in the presence of a specified amount of carrier $CaCl_2$ to give finally 0.03 μCi $^{45}CaCl_2$/ml cells suspension. During incubation at 37°C, duplicate samples were withdrawn, centrifuged at 4°C (30 s at 12 000 × *g*, Eppendorf centrifuge), and $^{45}Ca^{2+}$ was estimated in their supernatant. Ca^{2+} accumulation in the cells was calculated by subtracting the amount of $^{45}CaCl_2$ found in the supernatant of cells incubated with the ionophore from that of cells incubated without the ionophore [13].

Hemolysis determination. Cells were incubated with Ca^{2+} and ionophore, as described above. The reaction was terminated by cooling at 4°C, and the degree of hemolysis was determined on the supernatant at 540 nm.

Freeze-fracturing, thin-sectioning, and electron microscopy. Cells were fixed either at 37°C or at 4°C by addition of freshly prepared glutaraldehyde, pH 7.4 (Ladd Research, Inc.), to give a final concentration of 1% (v/v) in samples for freeze-fracturing, and 2% (v/v) in samples for thin-sectioning. Freeze-fracturing was performed as previously described [13]. Unless otherwise stated, etching time was 1 min. Samples for thin-sectioning were past-fixed in 1% OsO_4 and embedded in Epon 812. Thin sections were stained with uranyl acetate only.

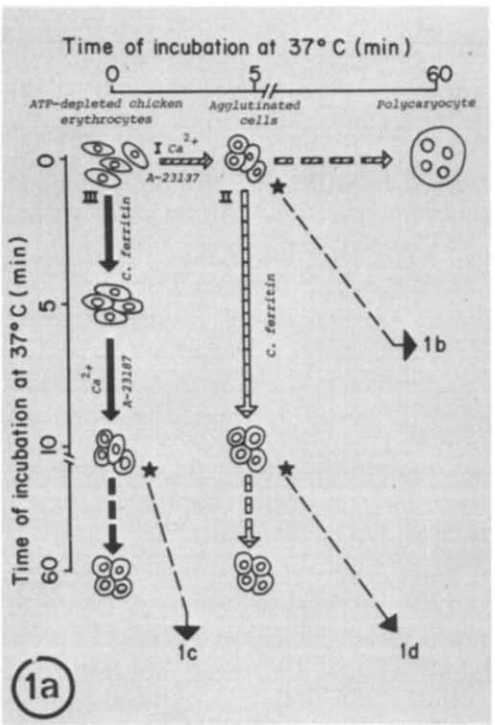

Fig. 1. Inhibition of fusion and cold-induced clustering of intramembrane particles by cationized ferritin. (a) A scheme describing the effect of cationized ferritin on Ca^{2+}- and ionophore-induced fusion of erythrocytes. ATP-depleted chicken erythrocytes (5%, v/v) were incubated for 5 min at 37°C and then

Electron micrographs were obtained with a Philips EM300 microscope operating at 80 kV (freeze-etching) and 60 kV (thin-sectioning).

Results

The effect of cationized ferritin on Ca^{2+}- and virus-induced membrane fusion and on the thermotropic separation of intramembrane particles in chicken erythrocytes

Fig. 1a summarizes schematically the effect of cationized ferritin on induction of membrane fusion by internal Ca^{2+} (Ca^{2+} and the ionophore A23187) in ATP-depleted chicken erythrocytes. Previously it has been shown that Ca^{2+} and the ionophore A23187 induced fusion in chicken erythrocytes [1,13]. Fusion was higher in ATP-depleted than in fresh cells [13]. Addition of cationized ferritin either before Ca^{2+} or to cells which were pre-allutinated by Ca^{2+} and the ionophore at 37°C, completely blocked formation of polykaryons. Membrane fusion did not develop in these systems even after 4 h incubation at 37°C (not shown).

Incubation in the cold of Ca^{2+}-containing cells induced extensive clustering of intramembrane particles (thermotropic separation), as was revealed after freeze-fracturing of the cells (Figs. 1b and 5a). Concomitantly with inhibition of membrane fusion, addition of cationized ferritin completely blocked the Ca^{2+}-induced thermotropic separation of intramembrane particles (Fig. 1c). Cationized ferritin inhibited cold-induced clustering of intramembrane particles when added either at zero time or after 5—10 min of incubation of chicken erthrocytes at 37°C with Ca^{2+} and ionophore, although in the latter case its inhibitory effect was less pronounced (Fig. 1d). Titration with increasing concentrations of cationized ferritin revealed that complete inhibition of membrane fusion, as well as of the thermotropic separation of intramembrane particles, was achieved with 180—200 μg of cationized ferritin per 1 ml cell suspension (5%, v/v).

Cationized ferritin also sharply inhibited and significantly delayed the onset of *Sendai* virus-induced fusion (Fig. 2), as well as the virus-induced thermotropic separation of intramembrane particles in chicken erythrocytes (not shown).

Incubation of Ca^{2+}-loaded fresh and ATP-depleted chicken erythrocytes at 37°C induced significant hemolysis of the cells. Hemolysis was smaller in fusible ATP-depleted cells than in fresh cells (Fig. 3). Cationized ferritin strongly reduced the Ca^{2+}-induced hemolysis in both fresh and ATP-depleted cells (Fig. 3).

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divided into three systems. $CaCl_2$ (30 mM) and the ionophore A23187 (20 μg/ml cell suspension) were added to systems I and II and the cells were incubated for 5 min at 37°C (▨). At this stage, strong agglutination and rounding of most of the cells could be observed. Then cationized ferritin was added to system II (▭) and both systems were further incubated at 37°C with gentle shaking (50 rev./min). Fusion of approx. 60% developed in system I, while no fusion was observed in system II. In system III, the cationized ferritin was added first (➡), and this was followed by the addition of $CaCl_2$ (20 mM) and a ionophore (20 μg/ml suspension). The cells were further incubated at 37°C as in systems I and II, and no fusion was observed. (b—d) Cold-induced clustering of intramembrane particles. At the points indicated by stars in a, samples were withdrawn, cooled at 4°C for 5 min, and then fixed and freeze-fractured as described in Materials and Methods. (b—d) ×60 000.

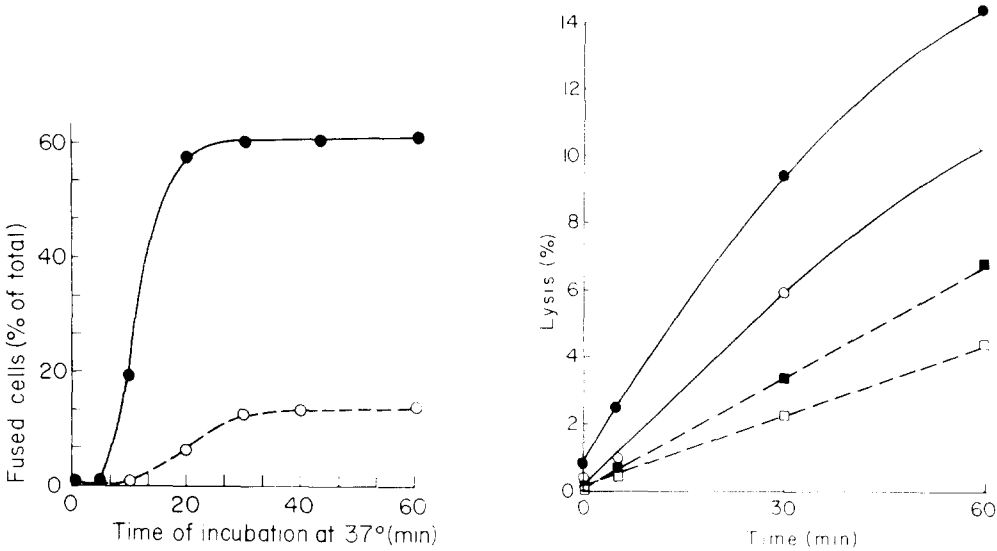

Fig. 2. Inhibiton of *Sendai* virus-induced fusion by cationized ferritin. Experimental conditions as described in Materials and Methods. The fusion process was initiated by the addition of *Sendai* virus (2000 HAU/ml cell suspension) to cells incubated at 37°C in the presence of either $CaCl_2$ (20 mM) (●———●) or both $CaCl_2$ and cationized ferritin (○- - - - -○). For estimation of the degree of fusion, samples were withdrawn, lysed rapidly in cold H_2O, immediately fixed in glutaraldehyde (1%, v/v), and then examined under a phase microscope. Lysing of the cells was necessary for visualization of nuclei in big clumps of cells agglutinated by cationized ferritin.

Fig. 3. Suppression of calcium- and ionophore-induced lysis in fresh and in ATP-depleted erythrocytes by cationized ferritin. Experimental conditions are identical to those described in Fig. 1a, systems I and III. Fresh (5%, v/v) (———) or ATP-depleted chicken erythrocytes (- - - - -) were incubated with $CaCl_2$ and ionophore in the presence (○, □) or absence (●, ■) of cationized ferritin.

It was important to study whether the inhibitory effect of cationized ferritin on induction of membrane fusion, hemolysis and thermotropic separation of intramembrane particles can be attributed to its effect on Ca^{2+} uptake into the cells. Fig. 4 shows that the same amount of Ca^{2+} was introduced into chicken erythrocytes by the ionophore A23187 in the presence or absence of ferritin particles.

Ca^{2+}- and virus-induced thermotropic separation of external anionic sites

The lateral movement of external anionic sites (as reflected by the movement of bound cationized ferritin molecules) could be studied by freeze-fracturing followed by deep-etching of the membranes or in thin sections.

As can be seen in Fig. 5, incubation in the cold of Ca^{2+}-loaded cells, besides promoting clustering of intramembrane particles, also induced aggregation of the external anionic sites. This could be assessed by visualization of large clumps of cationized ferritin formed on the surface of cooled cells (Fig. 5a). When cationized ferritin was added before cooling of the cells, it inhibited the cold-induced clustering of the anionic sites. Deep-etched membranes of these cells showed even distribution of the ferritin particles, both at 37°C (not shown) and after they were transferred to the cold (Fig. 5b). When cooled cells to which cationized ferritin is bound (see Fig. 5a) were transferred to 37°C, the

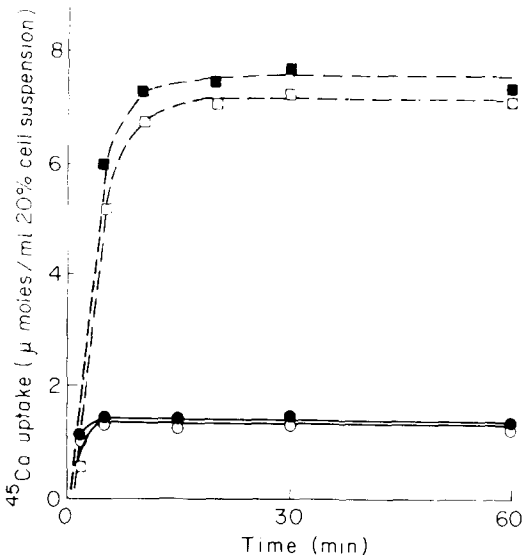

Fig. 4. Ca^{2+} uptake in ATP-depleted erythrocytes promoted by addition of the ionophore A23187 in the presence and absence of cationized ferritin. Experimental conditions as described in Materials and Methods. ATP-depleted chicken erythrocytes were incubated with 20 mM $CaCl_2$ (- - - - -) and 2 mM $CaCl_2$ (———) in the presence (□, ○) or absence (■, ●) of cationized ferritin and 20 μg/ml of the ionophore.

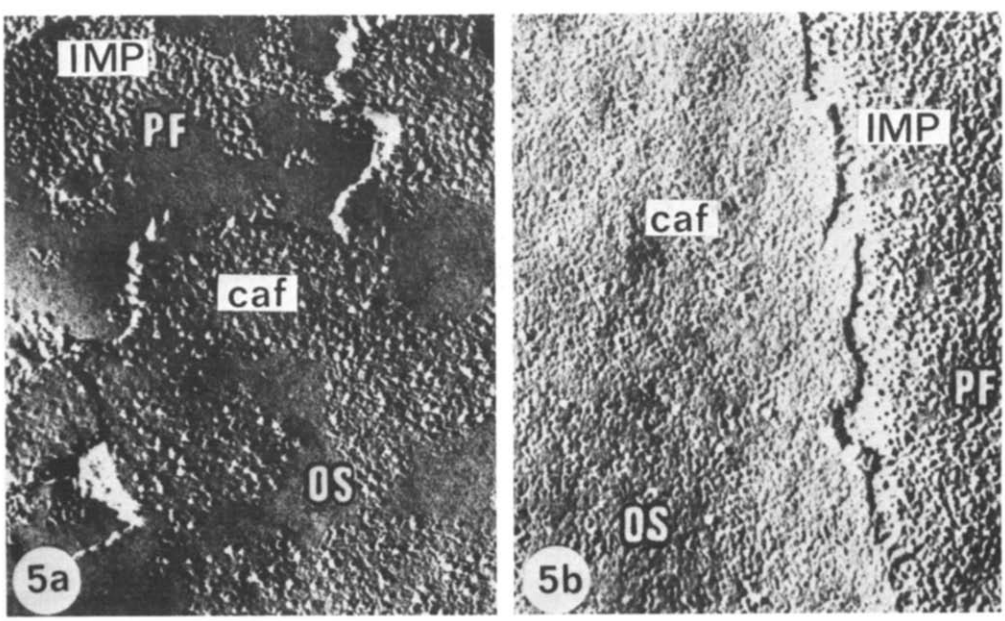

Fig. 5. Ca^{2+}-induced movement of cells' external anionic sites and its inhibition by cationized ferritin as visualized in fractured and deep-etched erythrocyte membranes. Fresh chicken erythrocytes (2.5%, v/v) were incubated at 37°C in the presence of $CaCl_2$ (2 mM). This was followed by two different treatments: (I) (a): After addition of the ionophore A23187 (2 μg/ml) followed by 10 min incubation at 37°C, the Ca^{2+}-loaded cells were transferred to 4°C for 5 min. Then cationized ferritin was added, and the cells were fixed in the cold for freeze-etching. Note in a the large aggregations of cationized ferritin (caf) on the external surface (OS) of deep-etched cells. Analogous clustering of intramembrane particles (IMP) could be seen on the protoplasmic fracture (PF) face of fractured membranes. (II) (b): The cationized ferritin was added at 37°C followed by the addition of the ionophore (2 μg/ml). After an additional 10 min at 37°C, the cells were cooled at 4°C for 5 min and prepared for deep-freeze etching. Note in b that the ferritin molecules (caf) seen on the external surface are evenly distributed, similarly to the intramembrane particles (IMP) on the protoplasmic fracture face of the underlying fractured membrane. In all cases the deep-etching was performed for 5 min. (a, b) X100 000.

intramembrane particles and the anionic sites underwent a process of redistribution and appeared scattered as in the control cells (not shown).

Aggregation of cationized ferritin on the surface of cooled Ca^{2+}-loaded cells could also be observed in thin sections of the cells (Fig. 6a). While cationized ferritin uniformly coated Ca^{2+}-loaded cells at 37°C (Fig. 6b), it was segregated into two distinct groups after incubation of the cells at 4°C (Fig. 6a). In Fig. 6a four cells (I—IV) aggregated by Ca^{2+} and the ionophore are seen.

The phenomenon of cold-induced clustering of external anionic sites was also observed during virus-induced agglutination and fusion of chicken erythrocytes. Even distribution of cationized particles was observed after it was added to cells incubated with virus for 2 min at 37°C (Fig. 7a). On the other hand, when cationized ferritin was added to these cells after being transferred to the cold, the ferritin particles were segregated into distinct patches (Figs. 7b and 7c). Virus particles were found attached to the cell surface only in regions

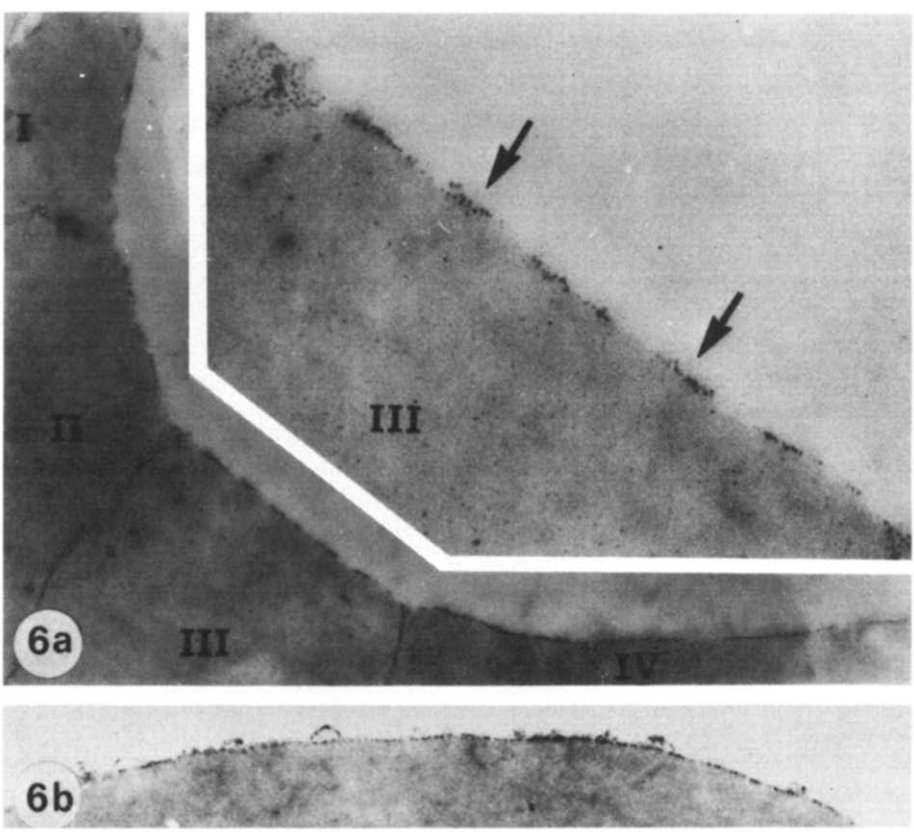

Fig. 6. Ca^{2+}-induced movement of cells' external anionic sites as visualized in thin sections. a shows a thin section demonstrating the same phenomenon which was shown in Fig. 5a by deep-etchin. Samples for thin-sectioning were taken at the same time as for deep-etching from the system described in Fig. 5a. Note the distinct clumps of cationized ferritin (arrows) on the outer surface of membranes of four different cells (I—IV) (×40 000). The intercept shows much higher magnification of cell III (×120 000). b, shows the distribution of cationized ferritin in cells treated as in a, except that the cells were not cooled (×60 000). Note the even distribution of ferritin particles on the cell surface.

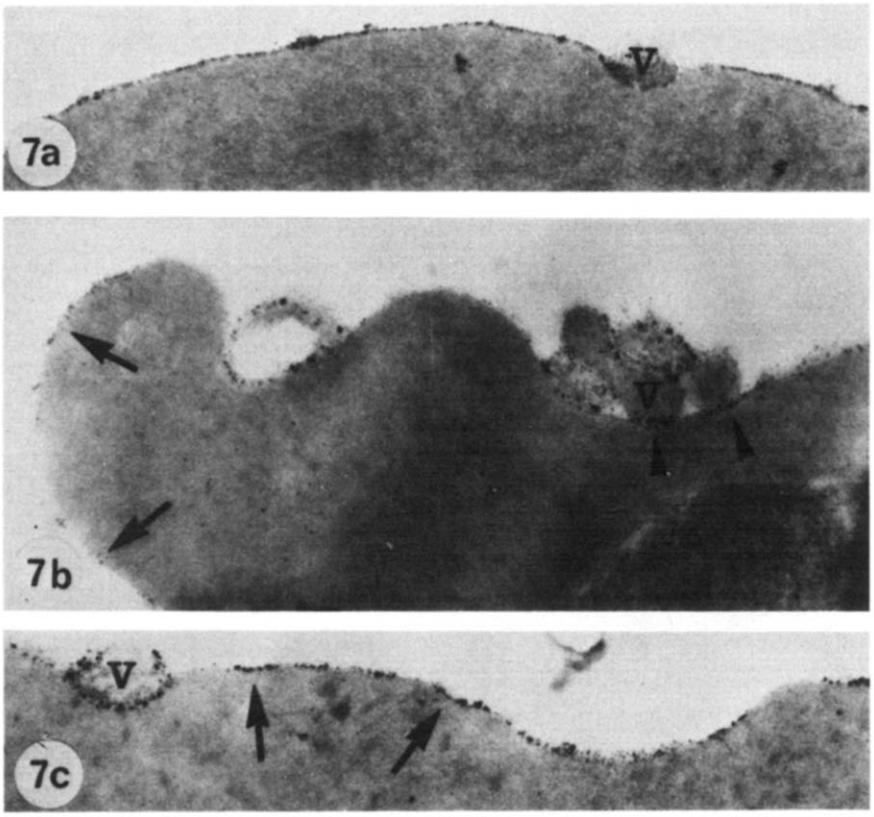

Fig. 7. Thermotropic separation of external anionic sites induced by the interaction of *Sendai* virus with erythrocytes; thin section demonstration. Chicken erythrocytes were incubated with *Sendai* virus as described in Materials and Methods. After 2 min at 37° C, the virus-agglutinated cells were either coated with cationized ferritin and fixed (a) or were first cooled at 4° C for 5 min * and then coated with ferritin and fixed in the cold (b and c). Distinct patches of ferritin particles on the cell surface (black arrows) can be easily seen in b and c. Note also that the ferritin label is clearly visible in most places of contact between the virus particle (V) and the cell membrane (black arrow heads). (a—c) X60 000.

which bound cationized ferritin particles, while they were completely absent from the ferritin-free regions (arrows, Fig. 7b).

Discussion

Cold-induced clustering of intramembrane particles was observed in several types of biological membranes, such as mitochondria [16], microsomes [18], and *Escherichia coli* [19]. This phenomenon probably depends on the composition of the membrane phospholipids and on the ability of membrane proteins to move laterally and in respect to each other. The failure to induced thermo-

* In order to ensure that viral effect on the distribution of intramembrane particles is not due to the passive accumulation of $CaCl_2$ in the cells in the cold, we used the following procedure (Volsky, D.J. and Loyter, A., unpublished): 0.5 ml cells were dropped into 14.5 ml cold solution K containing 2 mM ethyleneglycol bis(α-aminoethylether)-*N,N'*-tetraacetic acid. After cooling, cells were concentrated in the cold and coated with ferritin.

tropic separation of intramembrane particles in intact erythrocyte membranes [13] may be attributed to the fact that aggregation of membrqne proteins in these cells is controlled and restricted by the underlying spectrin network [8,9].

Based on the above view, we suggest that the cold-induced clustering of intramembrane particles observed previously [13,14] and in the present work in intact chicken erythrocytes, may occur only after full or partial removal of the motional restriction imposed on the integral membrane proteins by spectrin [8,9].

As was suggested before [20,21], the lateral movement of membrane proteins can be divided into at least two categories: (1) A long-range lateral diffusion, which is a streaming process involving transfer of intact membrane areas from point A to point B along the membrane. This kind of movement is unchanged during membrane fusion [20]. (2) A local lateral diffusion, i.e. diffusion restricted to short distances, This kind of diffusion, which promotes an increase in the lateral movement of proteins with respect to each other, could lead to protein aggregation either in the presence of Ca^{2+} [13], or at pH 5.5 in spectrin-depleted human erythrocyte ghosts [8].

The present work deals entirely with this kind of protein movement and its relation to the fusion process.

We suggest that an increase in the freedom of membrane proteins to move laterally with respect to each other, as reflected by their ability to undergo thermotropic separation, is a prerequisite for the initiation of the membrane fusion process, in accordance with the hypothesis that fusion occurs between regions of exposed phospholipids [21] *.

It is shown in the present work that the cold-induced clustering of intramembrane particles in chicken erythrocytes can be inhibited by cationized ferritin. This inhibitory effect seems to result from an externally mediated inhibition of movement of intramembrane particles, and not from interference with the membrane-modifying action of either *Sendai* virus or intracellular Ca^{2+}. The ionophore-induced Ca^{2+} accumulation as well as the rounding of cells remained unaffected. Moreover, cationized ferritin inhibited the thermotropic separation phenomenon also in cells which were loaded with Ca^{2+} prior to addition of ferritin (compare Fig. 1d with Fig. 1b).

Clustering of intramembrane particles was accompanied by similar and concomitant thermotropic separation of surface anionic (ferritin-labeled) sites, whose movement could also be blocked by cationized ferritin. Thus a structural correlation between surface anionic sites and the intramembrane particles seems possible. This was also suggested before [10].

It seems unlikely that the particles of cationized ferritin are bound to negatively charged phospholipids, as the latter are almost exclusively localized on the inner side of the membrane [22]. Conceivably the cationized ferritin is bond to negatively charged groups of externally located glycopeptides. Mem-

* It should be emphasized that very little or no change in the normal distribution of intramembrane particles was observed in the membranes of fusing cells at 37° C, as was reported by Fowler and Branton [20] and by us [13]. This is not surprising since the fusion area itself, from which proteins must be removed, is a limited and restricted region which is difficult to be exposed by freeze fracturing.

brane phospholipids are probably masked by surface glycopeptides, as is also reflected by the heavy, homogenous layer of cationized ferritin seen on the external surface of the cells. Blocking of the aggregation of the external anionic sites (and of the intramembrane particles) will thus prevent exposure of membrane phospholipids, a step which is required for the initiation of membrane fusion [21].

Indeed, as is shown in the present work, such an immolization of membrane proteins by cationized ferritin was accompanied by inhibition of Ca^{2+}- or virus-induced membrane fusion. A correlation between the ability of membrane proteins to move with respect to each other and the fusion was thus established. The above conclusion is strengthened by the observation showing that inhibition of virus-induced fusion is transient and partial. This is probably due to hydrolysis of membranes' sialic acid, to which the cationized ferritin is bound, by the viral neuraminidase [17]. Consequently, less ferritin particles are bound and the degree to which fusion is inhibited declines.

The observed blocking of membrane fusion cannot be due to inhibition of contact between opposing membranes by cationized ferritin. Cationized ferritin, as well as other polycation polymers like polylysine, are known to induce agglutination of red blood cels (refs. 17 and 24, and Fig. 1), and therefore should stimulate fusion rather than inhibit it [24]. Moreover, as is shown in the present work, cationized ferritin also inhibited cell fusion when added to cells already preagglutinated by Ca^{2+} and an ionophore (Fig. 1 and p. 217) where tight cell-contact was already established. In these cells, membranes in close proximity, without cationized ferritin, where membrane fusion can occur, could easily be found (not shown).

In the light of the results of the present work, it would be of interest to find out whether immobilization of intramembrane particles would inhibit other fusion processes, such as fertilization, conjugation in protozoa, and secretion of molecules from exocrine glands.

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