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REARRANGEMENT OF INTRAMEMBRANOUS PARTICLES AND FUSION PROMOTED IN CHICKEN ERYTHROCYTES BY INTRACELLULAR Ca^{2+} *

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Summary

Ca^{2+} was introduced into fresh and ATP-depleted chicken erythrocytes through the aid of the ionophore A-23187.

Intracellular Ca^{2+} (10—40 mM) induced fusion in ATP-depleted cells after 30—60 min incubation at 37°C, but not in fresh cells. Fresh cells underwent a higher degree of haemolysis than ATP-depleted cells after accumulation of Ca^{2+}. Uptake of Ca^{2+} was the same in these two systems.

Intracellular Ca^{2+} induced rearrangement of intramembranous particles, as revealed by freeze-etching studies. The intramembranous particles in the protoplasmic face of fractured membranes obtained from fresh cells incubated with 1 mM of Ca^{2+} were more scattered and their density was lower than in control cells. Incubation with higher concentrations of Ca^{2+} (10—40 mM) induced transient changes in the intramembranous particles' density with the appearance of protrusions and depressions on the protoplasmic and exoplasmic faces of the fractured membranes, respectively. These effects were reversible upon removal of Ca^{2+} by washing the cells with ethyleneglycol bis(α-aminoethylether)-*N*,*N*'-tetraacetic acid; rearrangement of intramembranous particles was less evident after accumulation of Ca^{2+} in ATP-depleted cells, whose fractured membranes did not contain any protrusions or depressions.

Transferring Ca^{2+}-loaded cells to the cold caused the formation of large smooth areas devoid of intramembranous particles in the protoplasmic face of the fractured membranes.

Cells containing Ca^{2+} appeared spherical, and removal of Ca^{2+} restored the normal oval shape of chicken erythrocytes.

* Preliminary results of this work were presented at the Sixth European Congress on Electron Microscopy (Jerusalem, September, 1976) [14].

Introduction

Bivalent cations, and especially calcium ions, were shown to be essential for promoting membrane fusion processes [1]. Virus-induced fusion of various cell lines is absolutely dependent on the presence of Ca^{2+} in the incubation medium, and addition of chelators, such as EDTA, prevents fusion completely [2]. Addition of Ca^{2+} is also essential for promoting membrane fusion by chemical fusogenic agents, such as glycerlymonooleate, oleic acid, or retinol [3]. Recently it has been reported that only in the presence of Ca^{2+} can phosphatidylserine-rich liposomes be fused between themselves, with eukaryotic cells, or even induce polykaryon formation [4—6]. Since phosphatidylserine is exclusively localized in the inner side of the erythrocyte membrane [7], it is conceivable that Ca^{2+} may affect membrane fusion, at least partially, by binding to phospholipids and/or proteins at the membrane side which faces the cytoplasm. This view is supported by experiments showing that when Ca^{2+} is introduced into chicken erythrocytes by the aid of the ionophore A-23187, fusion is induced and polyerythrocytes are formed [8]. In this case, however, internal Ca^{2+} was able to induce fusion either after removal of the membranes' sialic acid, followed by short incubation of these cells with Ca^{2+} and a ionophore at the high temperature of 47°C, or after 5 h incubation of non-treated Ca^{2+}-containing cells at 37°C [8]. Recent experiments in our laboratory showed that Ca^{2+} is able to induce fusion of intact human erythrocytes or ghosts prepared from them [9]. It appears that in this system also Ca^{2+} acts on the inner side of the human erythrocyte membrane [9].

It was suggested that fusion occured between membrane regions of exposed phospholipids from which proteins were removed [10]. Indeed, studies using the freeze-etching technique revealed that redistribution of intramembranous particles takes place when membranes are fused [11—14]. Bächi has observed clustering of intramembranous particles during fusion of human erythrocytes by *Sendai* virus [11]. Redistribution of intramembranous particles was shown in *Tetrahymena pyriformis* where mucocysts are fused with plasma membrane [12]; during secretion of histamine from zymogen granules in mast cells [13], or during virus-induced fusion of chicken erythrocytes [14]. It is noteworthy that in the last two cases Ca^{2+} is required for the fusion.

Redistribution of the intramembranous particles was observed also in spectrin-depleted human erythrocyte ghosts after addition of Ca^{2+}. and was suggested to be due to the Ca^{2+}-induced polymerization of the erythrocyte spectrin [15]. Interestingly, depletion of ATP from red blood cells of various species by respiratory inhibitors, also caused clustering of the intramembranous particles [16,17]. Assuming that clustering of the particles and exposure of membrane phospholipids is a prerequisite for the initiation of fusion, it might be postulated that fusion should be induced more rapidly in ATP-depleted cells than in fresh cells.

In the present work we have studied the effect of Ca^{2+} accumulation on the fusion ability of fresh and ATP-depleted cells. The changes induced in the normal pattern of intramembranous particles by internal Ca^{2+} in these systems were monitored and were correlated with the fusogenic ability of the internal Ca^{2+}.

Materials and Methods

Cells

Blood was collected from the necks of decapitated chickens into an Erlenmeyer flask containing Heparin (100 U/ml). The blood was kept in the cold and used within 3 days. Before the experiment, cells were washed (3 times) in Solnk (see Medium) and the buffy coat was carefully removed. The final sediment was suspended in Solnk to give 5% or 40% (v/v) concentration.

Medium

The medium used throughout the present work (Solnk) contained 130 mM KCl, 5.4 mM NaCl, 0.8 mM $MgCl_2$ in 20 mM tricine-NaOH (pH 7.4).

Introduction of Ca^{2+} into chicken erythrocytes by use of the ionophore A-23187

Chicken erythrocytes suspended in Solnk (5% or 40%, v/v) were incubated in the cold for 5 min with the indicated amount of the ionophore (between 10 and 30 μl of an ionophore solution of 0.3—3 mg/ml in ethanol were added to each ml of cell suspension) and then diluted with cold Solnk containing Ca^{2+} to give 2.5% or 20% (v/v) of cells. After an additional 5 min in the cold, the cells were incubated with gentle shaking at 37°C (New Brunswick shaking bath, 100 rev./min). In all experiments, except those where Ca^{2+} uptake was measured by use of ^{45}Ca, a cell suspension of 2.5% (v/v) was utilized.

Haemolysis 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 haemolysis was determined on the supernatant at 540 nm after centrifugation of the cells.

Measurement of Ca^{2+} uptake

Cells in Solnk (20% v/v) were preincubated with the ionophore (30 μg/ml) and the $^{45}Ca^{2+}$ (0.03 μCi/ml cell suspension) was added in the presence of specified amounts of carrier $CaCl_2$. After incubation at 37°C, the Ca^{2+} uptake process was terminated by centrifugation in the cold (3 min at 2000 rev./min) and duplicate samples (20 μl) were removed from the supernatant and added to toluene-Triton scintillation liquid for radioactivity estimation (Tri-carb liquid scintillation spectrometer, Packard).

Ca^{2+} uptake was calculated by subtracting the amount of Ca^{2+} found in the supernatant of cells incubated with ionophore (U) from control cells incubated without the ionophore (T = total). Amount of Ca^{2+} introduced specifically into the cells by ionophore was: In (intracellular Ca^{2+}) = $T - U$. The amount of Ca^{2+} which was absorbed non-specifically to the cell surface and trapped in the intracellular volume was estimated from the difference between the amount of total Ca^{2+} introduced into the system and that found in the supernatant of the cells incubated with Ca^{2+} in the absence of the ionophore. This non-specific absorption never exceeded 5—10% of the total $^{45}CaCl_2$, while the specific uptake ranged between 75% (with 2 mM Ca^{2+}) and 45% (with 20 mM Ca^{2+}) of the total $^{45}CaCl_2$ introduced into the system. Estimation of $^{45}CaCl_2$ uptake by

direct measurement of the radioactivity associated with the cells after incubation and centrifugation (see below) were in good agreement (±5%) with the above described method.

Measurement of intracellular distribution of $^{45}CaCl_2$ (free and membrane-bound)

$^{45}CaCl_2$ was introduced into the cells and its intracellular amount was calculated (*In*) as described above, except that $^{45}CaCl_2$ of 0.3 μCi was added to each ml of cell suspension. The pellet obtained was washed once with an equal volume of cold Solnk containing non-radioactive $CaCl_2$. The pellet obtained was haemolyzed by addition of 9 volumes of a hypotonic solution containing 5 mM $MgCl_2$ and 10 mM Tricine-NaOH, pH 7.4, and its total $^{45}CaCl_2$ estimated (*In'*). After centrifugation (15 min, 10000 rev./min) the amount of $^{45}CaCl_2$ in the clear supernatant was measured and was considered as free intracellular $CaCl_2$ (*L*). The amount of $^{45}CaCl_2$ associated with the pellet (membrane fraction = *M*) was estimated either directly, by counting samples obtained from it after solubilization in Soluene (Packard) : isopropanol (1 : 1) at 50°C for 1 h [16], or indirectly, by the following calculation: $M = In' - L$. The results obtained by the two methods were in good agreement (±5%).

ATP depletion and restoration

ATP was depleted and restored from chicken erythrocytes essentially as described before [16]. Briefly, washed erythrocytes (2.5%, v/v) were incubated for 8—12 h at 37°C with 1 mM KCN and 10 mM NaF. At the end of the incubation period the cells were washed 3—4 times in Solnk and resuspended to the desired concentration. Restoration of ATP was accomplished by incubating the washed ATP-depleted cells in Solnk containing 5 mM glucose, 1 mM adenine, 5 mM inosine, and 10 mM phosphate buffer (pH 7.4) at 37°C.

Freeze-etching and electron microscopy

Cells were fixed at a given temperature by addition of glutaraldehyde (Ladd Research Inc.) to give a final concentration of 1% (v/v) and then freeze-etching was performed, as described previously [16]. Micrographs were obtained with Philips EM 300 operating at 80 kV.

All reagent chemicals used were commercially obtained and were of analytical grade. The bivalent cation ionophore A-23187 was a gift from Eli Lilly and Co. (Indianapolis, Ind.) and was dissolved in ethanol (0.3—3 mg/ml). ^{45}Ca carrier free (2 mCi/ml) was from New England Nuclear, Boston, Mass.

Results

Ahkong et al. [8] have reported that prolonged incubation of desialized chicken erythrocytes at 37°C with Ca^{2+} and the ionophore A-23187 induced only low degree fusion. Indeed, as can be seen in Table I, incubation of fresh chicken erythrocytes up to 60 min at 37°C with increasing concentrations of Ca^{2+} and the ionophore, induced agglutination without promoting fusion. However, when ATP-depleted chicken erythrocytes were incubated at 37°C with 10—40 mM Ca^{2+} in the presence of a ionophore, large polykaryons were formed after 15—60 min (Table I). Incubation of cells, fresh or ATP-depleted,

TABLE I

FUSION OF ATP-DEPLETED CHICKEN ERYTHROCYTES BY INTRACELLULAR CA^{2+}

Cells of 2.5% (v/v) and ionophore at 30 μg/ml were used. All other experimental conditions as described in Materials and Methods.

System	Ca^{2+} (mM)	Agglutinated cells (% of total)	Fused cells (% of total) (time at 37°C, min)		
			15	30	60
Fresh cells	5—40	10—70	0	0	0
ATP-depleted cells	5	10	0	0	0
	20	50	5	10	20—40
	40	70	10	20—40	50—60

with high concentrations of Ca^{2+} (up to 40 mM) in the absence of ionophore, caused neither agglutination nor the formation of polykaryons. External Ca^{2+} (no ionophore added) did not promote fusion even after the cells were agglutinated by the agglutination agent, polylysine.

The uptake of Ca^{2+} promoted by the addition of the ionophore A-23187 in fresh and ATP-depleted cells was essentially the same and reached the same maximal value after about 10—30 min of incubation, whether the Ca^{2+} concentrations was 2 or 20 mM (Fig. 1). Uptake was also observed at 4°C, although this was slower than at 37°C. Approximately 1.5 μmol of Ca^{2+} were accumulated in cells incubated for 30 min with 2 mM Ca^{2+} and ionophore at 37°C, while 1.0 μmol of Ca^{2+} was introduced under the same conditions at

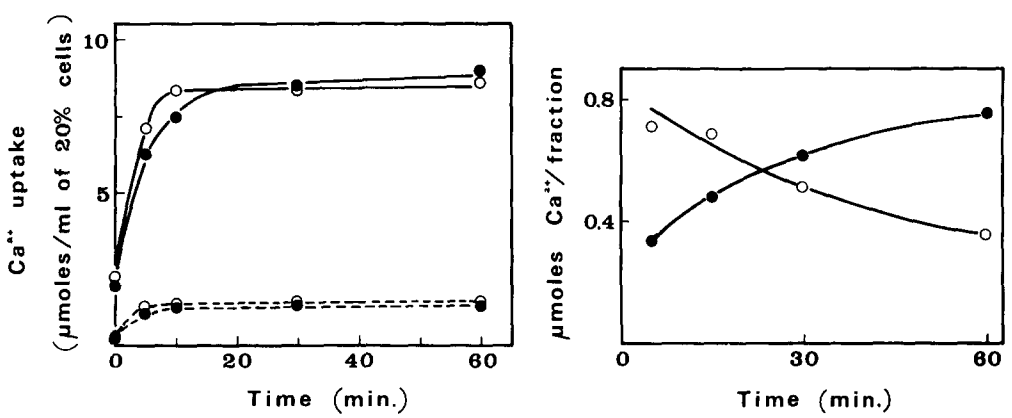

Fig. 1. Ca^{2+} uptake in fresh and ATP-depleted chicken erythrocytes promoted by addition of the ionophore A-23187. Experimental conditions as described in Materials and Methods. Fresh (○) and ATP-depleted (●) cells were incubated with either 2.0 mM $CaCl_2$ (- - - - -) or 20 mM $CaCl_2$ (———), and 30 μg/ml of the ionophore. The results in the figure represent one out of three separate experiments.

Fig. 2. Change in the ratio of intracellular-free and membrane-associated Ca^{2+} during incubation of chicken erythrocytes with Ca^{2+} and ionophore at 37°C. Ca^{2+} concentration, 2.0 mM; ionophore, 30 μg/ml. For experimental details see Materials and Methods. Each point represents the amount of Ca^{2+} found in 1 ml of lysate after lysis of 20% (v/v) cells, or the amount of Ca^{2+} associated with the membranes of 1 ml of 20% (v/v) cells. The data shown in the figure represent one of two independent experiments. ○———○, Unbound Ca^{2+} (lysate fraction); ●———●, bound Ca^{2+} (membrane-associated fraction).

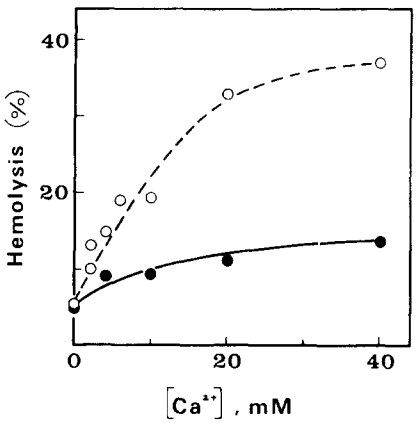

Fig. 3. Ca^{2+} and an ionophore induced hemolysis of the fresh and ATP-depleted chicken erythrocytes. Experimental conditions as described in Materials and Methods. Fresh (○- - - - -○) and ATP-depleted (●———●) cells were incubated for 30 min at 37°C with the indicated Ca^{2+} concentrations and 30 μg/ml of the ionophore.

4°C. As an uptake of 45—75% of the total $^{45}Ca^{2+}$ was observed (Fig. 1), it is suggested that Ca^{2+} is binding to intracellular sites, thus accumulating in these cells. The characteristics of such binding may be exemplified in Fig. 2.

Fig. 2 shows that the fate of intracellular Ca^{2+} was dependent upon the time of incubation at 37°C. Calcium was first accumulated in the cytosol of cells and was then gradually absorbed by the membranes. As can be seen in Fig. 2, out of a total amount of 1.1 μmol of Ca^{2+} accumulated in 1 ml of 20% (v/v) of chicken erythrocytes, approx. 0.8 μmol were found in the cytosol and 0.3 μmol were bound to the membranes after 5 min of incubation at 37°C. Further incubation at 37°C did not increase the total amount of intracellular Ca^{2+} (Figs. 1 and 2) but, after 60 min at 37°C, most of the intracellular Ca^{2+} was bound to the membranes' fraction (Fig. 2).

Accumulation of Ca^{2+} in chicken erythrocytes was accompanied by cell lysis, the degree of which was dependent upon Ca^{2+} concentrations (Fig. 3) and the time of incubation. Interestingly, Ca^{2+} induced a much higher degree of hemolysis in fresh than in ATP-depleted cells (Fig. 3). The lysis of fresh cells was increased almost linearly with increasing concentrations of Ca^{2+} up to 20 mM, while in ATP-depleted cells it leveled off at 2—4 mM of Ca^{2+} (Fig. 3).

The effect of intracellular Ca^{2+} on the distribution of intramembranous particles

The process of membrane fusion was shown to be accompanied by redistribution of intramembranous particles [11—14]. The technique of freeze-etching was used in the present work to investigate the differences between the membrane fractures of fusible ATP-depleted and unfusible fresh cells.

A profound decrease in the density of intramembranous particles was found in the inner face of membrane fractures (PF face) * of fresh cells incubated

* The freeze-etching nomenclature used throughout the present work is based on the recent proposition of uniform freeze-etching nomenclature, as outlined by Branton et al. [18].

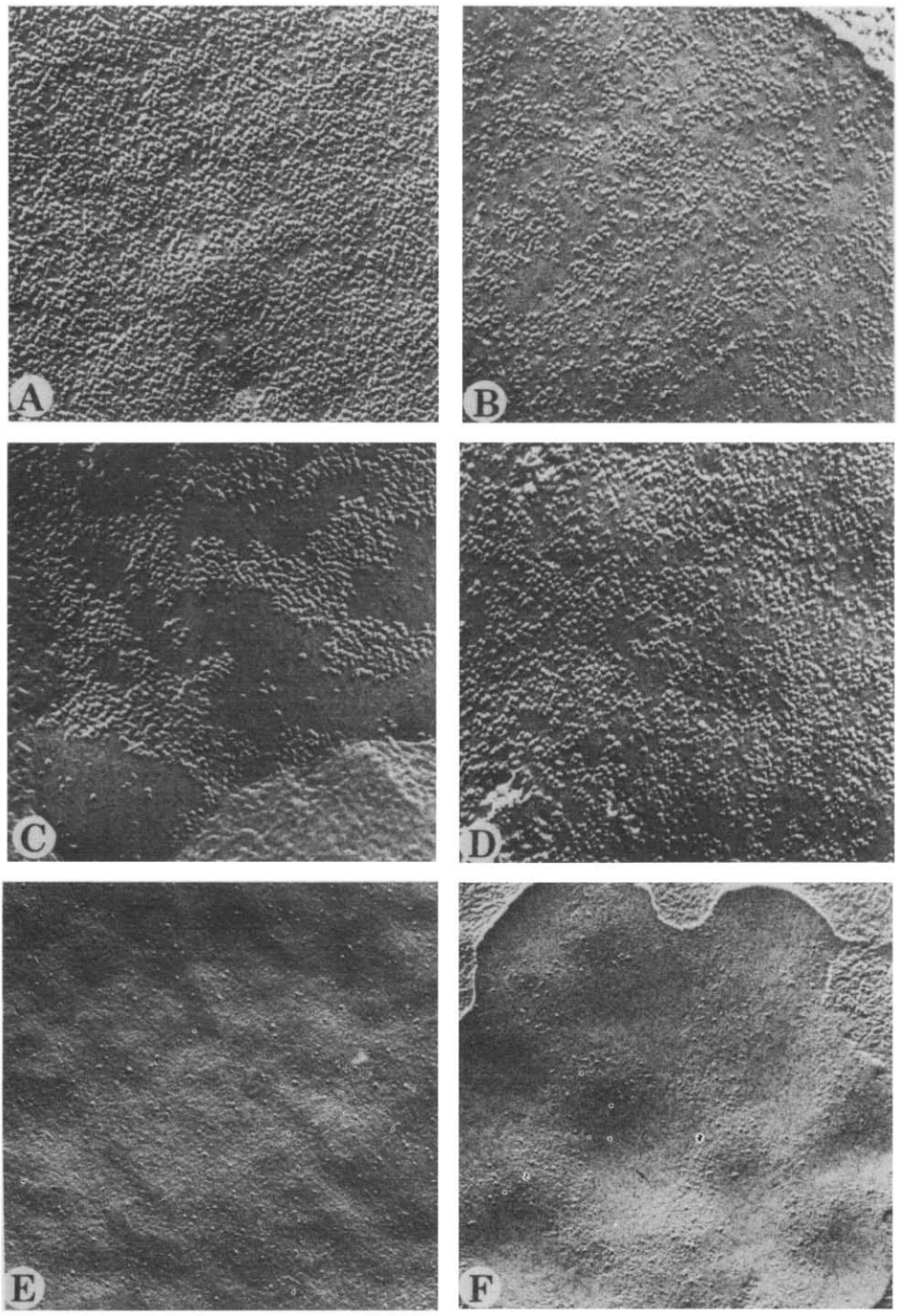

Fig. 4. Reversible changes in the distribution of intramembranous particles of freeze-fractured membranes of chicken erythrocytes incubated with 1 mM $CaCl_2$ and an ionophore at 37°C and at 4°C. Cells were incubated with $CaCl_2$ and ionophore as described in Materials and Methods. A—D. Replica of PF faces (×80000). A, 2 mM EGTA and 3 μg/ml ionophore, 30 min at 37°C; B, 1 mM $CaCl_2$ and 3 μg/ml ionophore, 30 min at 37°C; C, the system described in B transferred to 5 min at 4°C; D, C transferred back to 37°C for 5 min; E, EF face of control erythrocytes without any additions, 30 min at 37°C; F, as C, but showing EF face. E and F ×63000.

with 1 mM Ca^{2+} and the ionophore at 37°C (Fig. 4B and Table II). When these cells were transferred to the cold, an extensive clustering of intramembranous particles was induced (Fig. 4C). It can be clearly seen that the particles were segregated into several crowded areas separated by smooth particle-free regions. The cold-induced clustering of intemembranous particles was found to be reversible upon rewarming the cells to 37°C (Fig. 4D).

In the exoplasmic face (EF), the accumulation of Ca^{2+} also caused a decrease in the density of intramembranous particles (Table II). In addition, the particles of the EF face were clustered upon incubation of the cells in the cold (Fig. 4F). Titration of Ca^{2+} concentrations revealed that below 0.1 mM no changes in the intramembranous particles' distribution were induced.

It has been reported that depletion of ATP from chicken erythrocytes causes profound clustering of intramembranous particles [16]. Incubation of ATP-depleted cells with Ca^{2+} (1 mM) and the ionophore did not promote any further changes in the pattern of the particles or in their density (Table II) beyond that seen after ATP depletion. However, a reversible clustering of the particles was obtained by incubating Ca^{2+}-loaded, ATP-depleted cells in the cold and subsequent incubation at 37°C (not shown). Incubation of cells at 37°C or in the cold with ionophore alone or with the ionophore and ethyleneglycol bis-(α-aminoethylether)-*N*,*N*'-tetraacetic acid (EGTA) (2 mM) did not promote any redistribution of the intramembranous particles (see, for example, Fig. 4A).

A somewhat different picture was obtained when cells were incubated at high Ca^{2+} concentrations (10—40 mM) in the presence of an ionophore. Incubation of fresh cells with 10 mM Ca^{2+} for a short period of time (30 s) at 37°C

TABLE II

ALTERATIONS IN THE INTRAMEMBRANOUS PARTICLES' DENSITIES OF CHICKEN ERYTHROCYTE MEMBRANES, PROVOKED BY ACCUMULATION OF Ca^{2+} IN THE PRESENCE OF AN IONOPHORE

The numbers in the table represent average particle density of 8—10 micrographs. For each set of numbers the S.E. was calculated. The counting on individual micrographs was performed by random sampling of at least 30% of the total area of a micrograph of PF face and all the micrograph area of EF face. Thus, for each number presented in the table, about 6 000—18 000 particles were counted on the PF face, and 2 000—5 000 particles on the EF face. In most cases micrographs of final magnification of 150 000 were used. The systems described in the table are representative reproductions of experiments summarized in Figs. 4—6. Conditions of Ca^{2+} accumulation and freeze-etching are as described in Figs. 4—6 and in Materials and Methods.

System	Number of intramembranous particles per μm^2	
	PF face	EF face
Fresh cells		
Control, 30 min at 37°C	4263 ± 61	288 ± 7
1 mM $CaCl_2$, 30 min at 37°C	2860 ± 66	95 ± 7
1 mM $CaCl_2$ transferred to 4°C	2826 ± 70	91 ± 8
10 mM $CaCl_2$, 1 min at 37°C	3040 ± 117	127 ± 10
10 mM $CaCl_2$, 30 min at 37°C	6359 ± 54	446 ± 7
40 mM $CaCl_2$, 30 min at 37°C	6186 ± 126	490 ± 16
ATP-depleted cells		
Control, 30 min at 37°C	3910 ± 111	222 ± 8
1 mM $CaCl_2$, 30 min at 37°C	3778 ± 70	220 ± 10
40 mM $CaCl_2$, 30 min at 37°C	4930 ± 116	276 ± 7

caused, in addition to the reduction in intramembranous particles' density, the appearance of numerous small protrusions (Fig. 5A). Further incubation at 37°C (30 min) caused a drastic increase in the particles' density and they appeared tightly packed (Fig. 5B). Protrusions which were almost completely devoid of intramembranous particles were distributed in the PF face of these cells. Survey of large numbers of freeze-fractured membranes showed that the number of protrusions increased with increasing Ca^{2+} concentrations up to 40 mM (compare Fig. 5B with Figs. 6A and B). Cooling to 4°C of cells preincubated with high concentrations of Ca^{2+} (10—40 mM) caused the appearance of large smooth areas devoid of intramembranous particles. The protrusions in the membranes of these cells were localized in regions containing the particles (Fig. 5C). The EF face of these cells showed many depressions, probably the indentations left by the protrusions seen in the PF face of these membranes (Fig. 5E). The particles in the EF face shown in Fig. 5E are arranged around the depressions (compare to Fig. 4F). A higher magnification of this fracture is presented in Fig. 5F. The intramembranous particles in these membranes are heterogeneous with respect to size and height, as opposed to the homogeneous and evenly distributed particles in the EF face of cells incubated at 37°C only (compare with Fig. 4E).

Removal of Ca^{2+} from the cells by successive washing with EGTA caused complete disappearance of the protrusions and depressions from the PF and the EF faces of the fractured membranes, respectively. The appearance and the distribution of the intramembranous particles in washed cells was similar to that in the control, untreated cells. Incubation of the EGTA-washed cells at 4°C induced, however, formation of smooth areas (Fig. 5D).

Freeze-fractured membranes of Ca^{2+}-loaded, ATP-depleted cells exhibited a different picture. Incubation of ATP-depleted chicken erythrocytes with high concentration of Ca^{2+} (up to 40 mM), in the presence of a ionophore, induced a smaller increase in the intramembranous particles' density as compared to fresh cells (Table II and Fig. 6C). Moreover, the large number of protrusions and depressions seen in the fractured membranes of fresh cells incubated with 40 mM Ca^{2+} (Fig. 6A and B) were completely absent in ATP-depleted cells (Fig. 6C). The prolonged incubation (6—12 h) of Ca^{2+}-loaded, fresh erythrocytes at 37°C, which results in ATP depletion [19], caused gradual disappearance of the protrusions and depressions in the fractured membranes as well as the appearance of fused cells. These changes did not occur when Ca^{2+}-loaded cells were incubated at 37°C with an ATP-restoration medium (not shown).

Table II is a quantitative summary of the changes observed in the number of intramembranous particles per μm^2 after incubation of chicken erythrocytes with Ca^{2+} and a ionophore. The data presented in this table clearly show that incubation of fresh cells at 37°C with either 1 mM Ca^{2+} for 30 min or with 10 mM Ca^{2+} for 1 min caused a significant reduction (approx 30%) in the number of particles per μm^2 in the PF face. Subsequent incubation at 37°C with 10 mM Ca^{2+} reversed the effect, and the density of the particles greatly increased. Under these conditions most of the cells became rounded and appeared as spheres (Fig. 7). The density of the particles in the PF face of ATP-depleted cells was not reduced by incubation with 1 mM Ca^{2+}. However, an increase in the density was observed upon incubation of cells with 40 mM Ca^{2+} at 37°C

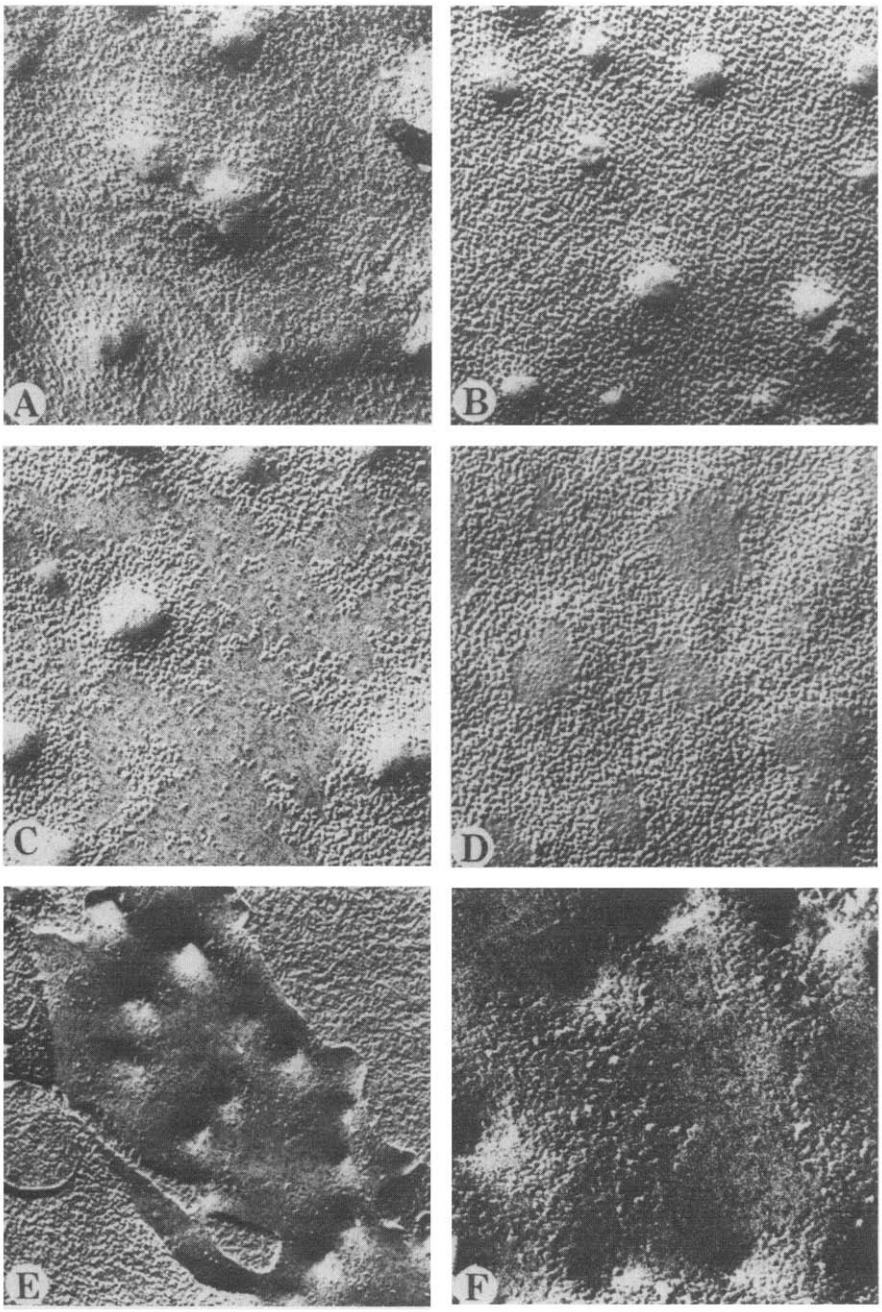

Fig. 5. Reversible reduction in intramembranous particles' density and the appearance of protrusions and depressions upon incubation of chicken erythrocytes with 10 mM Ca^{2+} and an ionophore. A—D. Replica of PF face (×80000). A, 30 s at 37°C; B, 30 min at 37°C; C, B transferred to 4°C for 5 min; D, B washed 5 times with 25 volumes of 0.2 mM EGTA, incubated for 10 min at 37°C and transferred to 4°C; E, EF face of C (×63000); F, as E (×150000). Ionophore concentration used: 5 μg/ml.

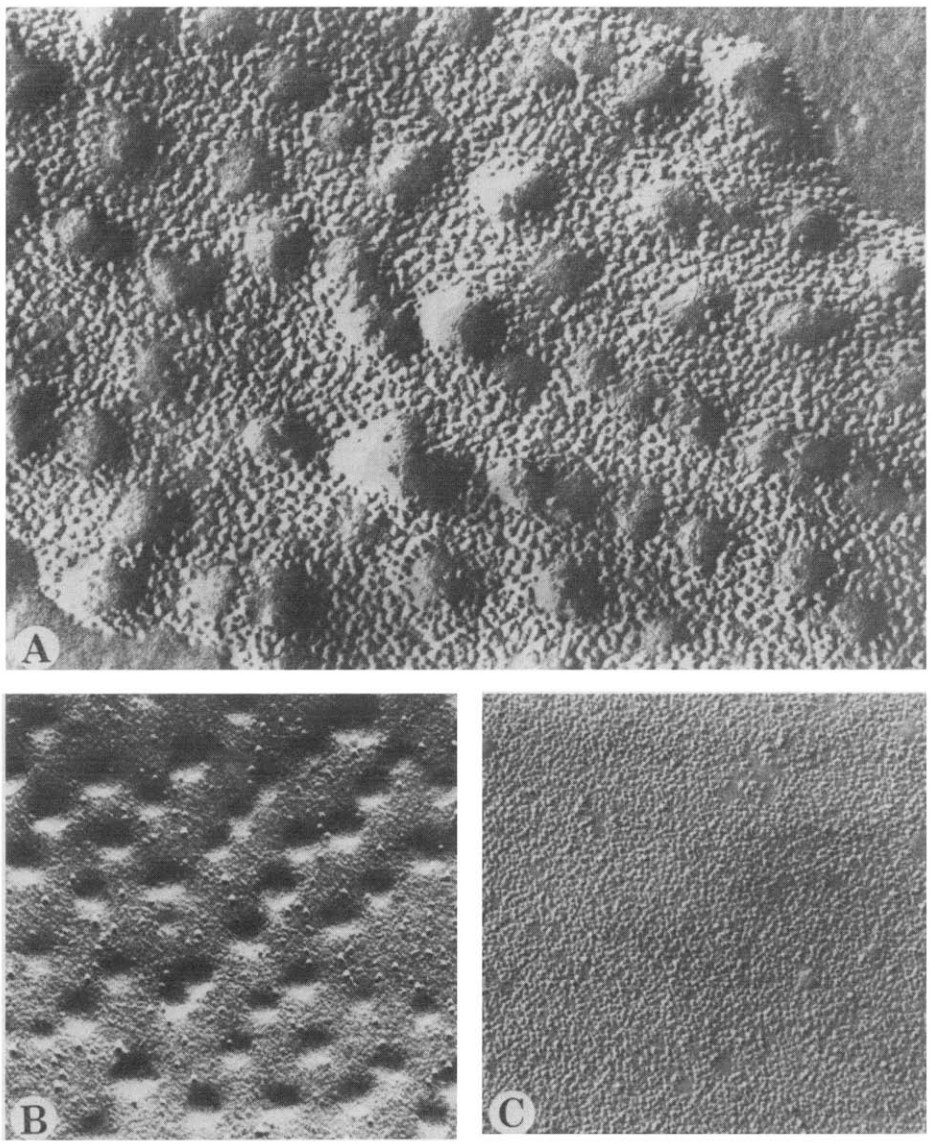

Fig. 6. Comparison between freeze-fractured membranes of Ca^{2+}-loaded, fresh and ATP-depleted cells. A. PF face of fresh cells incubated with 40 mM Ca^{2+} and 30 μg/ml of ionophore during 30 min at 37°C (×110000); B, as A but the EF face (×80000); C, PF face of ATP-depleted cells incubated as in A (×80000).

(Table II). Essentially the same pattern of changes in the density of intramembranous particles was observed in the EF face of the membranes.

Ca^{2+}-induced reversible changes of chicken erythrocytes' morphology

Fig. 7 shows that intracellular Ca^{2+} induced changes in the morphology of chicken erythrocytes causing the formation of round cells, as has already been reported before [8]. Both fresh and ATP-depleted cells became rounded upon

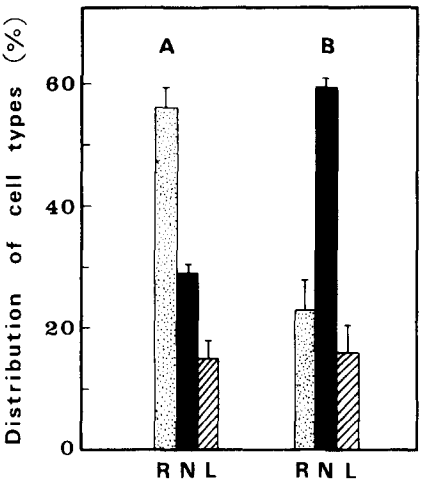

Fig. 7. Quantitative representation of the reversible changes in the shape of chicken erythrocytes induced by accumulation and removal of Ca^{2+}. Ca^{2+} (10 mM) was accumulated in fresh chicken erythrocytes (2.5%, v/v) in the presence of 5 μg/ml ionophore for 20 min at 37°C. At the end of the incubation period, samples were removed for observation in the phase microscope and counted (A). The rest of the cell suspension was centrifuged and the pellet was washed 5 times with 25 volumes of Solnk containing 0.2 mM EGTA. After the washings, during which about 10% of the total cell number was lost, the final pellet was suspended in Solnk to give 2.5% (v/v) and was then incubated for 20 min at 37°C, after which samples were again removed for observation in the phase microscope and counted (B). The cells seen in the microscope's field were divided into three main groups, as follows: Normal-shaped cells (N), rounded-shaped cells (R), and hemolysed cells, in which free nuclei or cells containing low amount of hemoglobin were counted (L). The data presented in the figure are an average of three independent experiments in each of which about 700 cells were counted and their shape distribution calculated. The data given in the figure, therefore, represent in each case (A or B) results obtained after counting a total of 2100 cells.

incubation with Ca^{2+} and the ionophore. Rounding of cells was also induced during incubation of cells with Ca^{2+} and ionophore in the cold. About 55% of the cells became rounded after 20 min incubation at 37°C in the presence of 10 mM of Ca^{2+} and the ionophore. The number of rounded cells decreased from 55% to about 20%, with the proportional increase in the normally shaped cells, after washing of cells with EGTA. At longer periods of incubation at 37°C or after raising the Ca^{2+} concentration (>20 mM), a higher percentage of cells (80—90%) became rounded. Under these conditions, only a small fraction of the rounded cells could regain its normal shape after washing with EGTA. These results indicate that, after a certain stage, rounding is irreversible. This might be due to a net decrease in the surface area of the plasma membrane caused by a pinching off of protein-free membranous vesicles [15,20,21].

Discussion

The extensive work of Branton's group on the ultrastructure of the human erythrocyte membrane [15,22,23] strongly suggests that the distribution of the intramembranous particles is regulated by the complex spectrin-erythrocyte actin (band 5). Recently Yu and Branton have shown that the polypeptides designated as band 3 are the main constitutent of the intramembranous particles [23] and that a recombinant formed between band 3 and phospholipids is

able to specifically bind spectrin and erythrocyte actin. Since polypeptides' bands similar to spectrin, band 3 and actin (band 5) of human erythrocyte membranes were also observed in the plasma membrane of avian erythrocytes [24,25], it is conceivable that their interrelationship is similar to that suggested for human erythrocyte membranes.

Table III summarizes and Fig. 8 illustrates the main observations of the present work which are the following: (1) Accumulation and release of Ca^{2+}-induced reversible morphological changes (oval → spheroid → oval) in the shape of chicken erythrocytes. (2) Introduction of Ca^{2+} into chicken erythrocytes caused alterations in the membrane ultrastructure, as revealed by the freeze-etching technique. (3) High degree of fusion was promoted by intracellular Ca^{2+} after relatively short periods of incubation in ATP-depleted cells (Fig. 8, III') but not in fresh cells (Fig. 8, III and IV).

Most interestingly, incubation of Ca^{2+}-loaded cells at low temperature induced drastic changes in the normal density and pattern of intramembranous particles. The alterations observed are most likely due to thermotropic separation between the bilayer lipid and integral proteins which are expressed as intramembranous particles. Binding of the intracellular Ca^{2+} to the phospholipids located on the inner side of the plasma membrane may induce segragation of the membrane phospholipids into different domains of different phase transition temperatures, as was demonstrated in the case of pure phospholipids and was suggested to be the mechanism by which Ca^{2+} promotes fusion of pure phospholipid liposomes [5,26,27]. Similar cold-induced changes in the pattern of intramembranous particles were demonstrated in membranes of *Escherichia coli* [28], mycoplasma [29] and mitochondria [30]. However, the aggregation of the particles, as observed in the present work, would also imply a cold-induced partial dissociation of the intramembranous particle-spectrin bond,

TABLE III

A SUMMARY OF THE STRUCTURAL CHANGES OBTAINED AFTER INTRODUCTION OF Ca^{2+} INTO CHICKEN ERYTHROCYTES

System	Morphology	Particle/μm^2		Appearance of PF face of cells incubated at 4°C	Protrusions (small blebs)	Fusion
		37°C	4°C			
Fresh cells	Oval	4200	4200	Normal distribution	—	—
Fresh cells + 1 mM Ca^{2+}	30—40% of cells are rounded	2860	2826	Clustering of particles	—	—
Fresh cells + 10 mM Ca^{2+}	All cells are rounded	6350	3500	Appearance of huge smooth areas	+	—
ATP-depleted cells	Oval (swollen)	3900	3850	Partially clustered	—	
ATP-depleted cells + 1 mM Ca^{2+}	30—40% of cells are rounded	3800	3750	Clustering of particles	—	—
ATP-depleted cells + 10 mM Ca^{2+}	All cells are rounded	4900	Not done	Not done	—	+

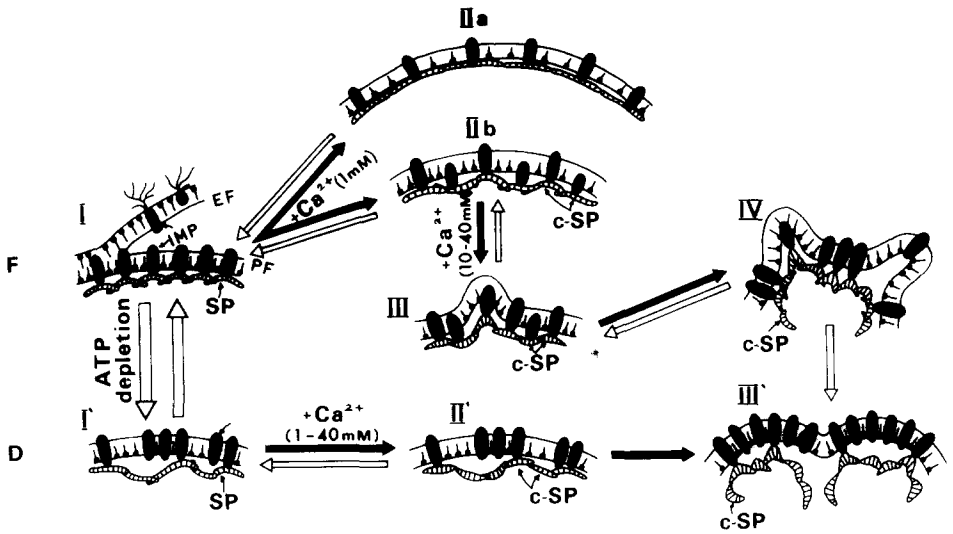

Fig. 8. Scheme explaining the ultrastructural and morphological alterations observed in intact and ATP-depleted chicken erythrocytes after Ca^{2+} accumulation in the presence of a ionophore. Incubation of chicken erythrocytes with low concentrations of Ca^{2+} (1 mM) and the ionophore A-23187 caused a decrease in the intramembranous particles' density (IIa and IIb). The decrease in the particles' density may be due to either expanding of the cell surface area (IIa) or to the disappearance of the particles from the membrane surface due to Ca^{2+}-induced polymerization of spectrin (IIb). At higher Ca^{2+} concentrations (10—40 mM), small protrusions have been found (III) whose number was increased with the time of incubation or with increasing Ca^{2+} concentrations (IV). The intramembranous particles' density increased after prolonged incubation (IV) because of shrinkage of cells and pinching off of protein-free membrane vesicles. In ATP-depleted cells, whose intramembranous particles are initially partially clustered (I') (cf. ref. 16), accumulation of Ca^{2+} did not have any significant effect on the pattern of the particles (II'). However, ATP-depleted, Ca^{2+}-loaded cells are fused after incubation at 37°C (III'), probably due to a weakening of membrane protein-phospholipid interaction [16]. When fresh, Ca^{2+}-loaded erythrocytes were submitted to prolonged incubation at 37°C (6—12 h), the number of protrusions decreased until they eventually disappeared, and the cells fused (IV → III'). F, fresh cells; D, ATP-depleted cells; PF and EF, the protoplasmic and the exoplasmic fractured faces of the membranes, respectively [18]; c-SP, contracted (polymerized) spectrin; IMP, intramembranous particles.

allowing the particle to move. Thus, it is conceivable that intracellular Ca^{2+} affects both the state of the lipid bilayer and the mutual interaction between the intramembranous particles and the spectrin network.

The density of the intramembraneous particles in the PF face of freeze-fractured membranes obtained from fresh cells incubated with low concentration of Ca^{2+} (1 mM) was lower than in control cells (Fig. 8, II). We would like to suggest that the effect of Ca^{2+} on the spectrin skeleton and its association with the intramembranous particles (band 3) can explain the above phenomena. The polymerization of spectrin induced by 1 mM Ca^{2+} would impose a vertical movement on the particles, removing them from the membrane to where they cannot be visualized by the freeze-etching technique (Fig. 8, IIb). In this context, it should be mentioned that recently, based on fluorescence measurement, a vertical movement of human surface proteins have been suggested [31].

The reversible alterations in the intramembranous particles' density upon incubation with Ca^{2+} may also be explained by changes in the cell volume. Microscopic observations revealed that a morphologically intermediate stage, at whcih cells are partially oval and swollen, occurs between the shrunken spheroid shape obtained after introduction of Ca^{2+} and the normal oval shape

(Fig. 7). The rise in the cell volume would cause an expansion of the plasma membrane and the stretching of the spectrin network. The resulting increase in the molecular distance between membrane proteins (and phospholipids) would cause the observed decrease in intramembranous particles' density (Fig. 8, IIa). However, the plasma membrane should be refractile to expansion due to the underlying cytoskeleton; therefore, the former mechanism of vertical movement seems more likely. In addition, specific conformational changes in the membrane proteins or glycoproteins may contribute to the reduction observed in the particles' density.

The protrusions seen at higher Ca^{2+} concentrations may be the result of lateral pressure imposed from the internal part of the cell (Fig. 8). This may be created by contraction and decrease in the surface area of spectrin due to its further polymerization by the higher intracellular Ca^{2+} concentrations [15]. As a result, small blebs, which are probably protein-free areas, would be protruded from the membranes (Figs. 5, 6, and 8, III and IV). It is very difficult to follow the fate of the intramembranous particles under such conditions. One of the most attractive assumptions would be that the particles remain attached to spectrin, whose network would lie beneath the surface of the protrusions. Indeed, most of the protrusions were devoid of intramembranous particles. Occasionally, a few particles, due to their strong association with the membrane phospholipids, will be detached from the spectrin-actin complex and appear on the surface of the small protrusions. Close examination of the protrusion surface demonstrates that a few of them possess a small number of intramembranous particles. The high increase in the number of particles per μm^2 found in the spheroid-shaped cells after prolonged incubation with Ca^{2+} at 37°C, is probably due to shrinkage of cells after decrease in the cell volume by Ca^{2+}-induced release of K^+ and water [21,22,32]. This is accompanied by reduction in the cell's surface area, occurring primarily by the pinching off of protein-free vesicles [15,21,22].

In ATP-depleted cells, in which protrusions are not formed, Ca^{2+} either fails to polymerize spectrin or, alternatively, the polymerized spectrin is partially dissociated from the intramembranous particles and does not control their movement (Fig. 8, I′—III′). This may explain the results showing that Ca^{2+} and ionophore induce fusion in ATP-depleted cells but not in fresh cells. Recently we have observed that *Sendai* virus, which induces membrane fusion, causes a rapid and specific dephosphorylation of phosphorylated membrane proteins [33]. Furthermore, we have shown that the combination of Ca^{2+} and phosphate will induce fusion only in ATP-depleted human erythrocytes [9]. It is conceivable that the first step in the fusion reaction is dephosphorylation of membrane proteins, thus releasing the intramembranous particles from their restraint. The involvement of spectrin with the fusion process was inferred from work in our laboratory [34] and that of Lucy's [35].

Fusion of chicken erythrocytes by various fusogenic agents, including either viruses of the paramyxovirus group or non-viral chemical agents, is always preceded by rounding of cells destined to fuse [3,8,36]. It is noteworthy that the first visible effect of internal Ca^{2+} on chicken erythrocytes is the induction of rounding of the cells. The oval shape of chicken erythrocytes is stabilized by the cell microtubules which are organized in the form of "marginal bands"

lying in a plane parallel to the flat surface of the cells [37]. It is thus conceivable that the rounding of cells observed after Ca^{2+} accumulation may be due to Ca^{2+}-induced disorganization of these microtubules [38]. The restoration of normal shape after removal of the internal Ca^{2+} and subsequent incubation of the cells at 37°C (Fig. 7) may be attributed to reassembly of the depolymerized microtubules. The fact that chicken erythrocytes become rounded before fusing may indicate that dissociation of microtubules is a necessary step for allowing nucleated erythrocytes or other eukaryotic cells to fuse. A correlation between the process of cell fusion and the organizational state of the cell microtubules is thus suggested. Experiments to study this possibility are currently under way in our laboratory.

From the observations of the present work we suggest that the role of Ca^{2+} in promoting cell fusion is dual. First, it accumulates in the cytosol and, by binding to microtubules, it causes their disassembly. This result in changes of the cell shape and allows mixing of the cell content after fusion. Second, by direct binding to the membrane phospholipids and proteins, it causes a change in their state (the separation of phospholipids into domains and polymerization of spectrin in the case of erythrocytes). This will induce cell fusion in ATP-depleted cells in which membrane phospholipids are weakly associated with the membrane proteins [39].

During the preparation of the present manuscript, a work showing the effect of low concentrations of intracellular Ca^{2+} on the normal distribution of intramembranous particles in chicken erythrocytes has appeared [40].

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