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CHEMICALLY INDUCED FUSION OF FRESH HUMAN ERYTHROCYTES

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SUMMARY: The fusion of fresh human erythrocytes was shown to be induced by calcium and phosphate ions. Prior treatment of erythrocytes with phosphate ion was a pre-requisite for the calcium-induced fusion. ATP levels in cells incubated with phosphate and calcium decreased 46 fold while cell-associated calcium increased 70 fold during 1 hour of incubation at 37°C as compared to cells which were incubated with calcium in saline. Our results suggest that a phosphate complex formed bridges between adjacent erythrocytes causing agglutination followed by aggregation of membrane proteins leading to protein-free areas of lipids. Where these protein-free areas are in close contact fusion may occur.

INTRODUCTION: The mechanism of cell fusion is of interest because fusion is common to many biological events, such as mitosis, phagocytosis, fertilization, virus entry into cells, etc. Fusion in vitro was first carried out using Sendai virus (1) as a fusing agent and for several years most investigators using cell fusion as a tool used Sendai virus. During the past ten years other fusing agents (2-5) have been described. Such studies of chemically induced fusion have renewed interest in the mechanism of fusion because of the apparent simplicity of the chemical approach as compared to the complexity of virus-induced fusion.

The fusion of human red blood cell ghosts by the combined action of calcium and phosphate ions has been described (6). The authors reported that fusion by these agents is ATP-dependent and that fresh intact cells agglutinated in the presence of phosphate and calcium did not fuse. We report here the fusion of fresh ATP-replete intact human red cells by calcium and phosphate with concomitant measurements of calcium influx, ATP levels, percent

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Abbreviations: DIDS; 4,4'-diisothiocyano-2,2'-stilbene disulfonic acid; PBS phosphate buffered saline.

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hemolysis and fusion index. Our studies show that interaction of phosphate ion with the external surface of cell membrane is a prerequisite in the fusion of cell membrane induced by calcium ion.

MATERIALS AND METHODS: Blood was drawn in heparin from human volunteers and used on the same day. It was washed 3x in saline (pH 7.0, 300 mOsm) and resuspended in 10-44 mM phosphate buffered saline (11 mM KH_2PO_4; 60 mM Na_2HPO_4; 147 mM NaCl, pH 7.4). Appropriate volumes of 80 mM $CaCl_2$ in saline were added to make a final concentration of 2-20 mM and cell volume adjusted to 10% PCV. Alternatively, after 10 minutes in phosphate buffered saline, the cells were centrifuged and the supernatant was discarded. To the pellet $CaCl_2$ (2-20 mM) in saline was added and volume adjusted to a hematocrit of 10%. After 10 minutes at room temperature, the cell suspension was further incubated at 37°C for varying periods of time.

ATP content in the cell was measured by the firefly method of Stanley and Williams (7). Calcium was assayed by atomic absorption spectrophotometry (Perkin-Elmer, Model 290) using an air-acetylene flame. Cells were prepared for analysis by digestion with 5 volumes of 10% nitric acid (Ultrex, J. T. Baker). Lanthanum oxide (0.5%) was added to all specimens and standards to inhibit phosphate interference. The measured volumes were corrected for calcium loss resulting from hemolysis during the washing steps.

Freeze-fracture was done with a Polaron unit (Polaron Corp., Line Lexington, PA). Freezing of a 25% glycerol suspension of glutaraldehyde-fixed cells was in Freon 22 at -150°C.

Washed cells were pre-treated with 2-10 μM, 4,4'-diisothiocyano-2, 2'-stilbene disulfonic acid (DIDS) for 15 minutes at 4°C to inhibit transport of phosphate ion (8).

Fusion indices (9) were calculated from the relation $F.I.=N_o / N_t - 1$, where N_o= number of cells at time 0, N_t= number of cells after incubation for time t. N_o and N_t were counted in a Model A Coulter Counter with a 70 μm aperture.

Percent hemolysis was measured by hemoglobin absorption on a Klett colorimeter or Cary 14 spectrophotometer at 415 nm.

RESULTS: Freshly drawn human red cells suspended in phosphate-buffered saline (PBS, 2-20 mM) agglutinated in a few minutes when calcium (2-20 mM) in saline was added. After 10 min. at room temperature, the cells were incubated at 37°C for varying periods of time. Fusion was typically observed after 20-30 min. at 37°C (Fig. 1) In agreement with a previous report on the fusion of ghosts (6) the addition of cells to a mixture of calcium and phosphate was ineffective in causing agglutination, hemolysis or fusion. Neither calcium or phosphate alone caused agglutination.

As a check on the importance of a visible precipitate of calcium phosphate to the fusion process, red cells resuspended in phosphate buffer were centrifuged and the supernatant removed. Then calcium in saline was added to the pellet. On vortexing this mixture the cells immediately agglutinated

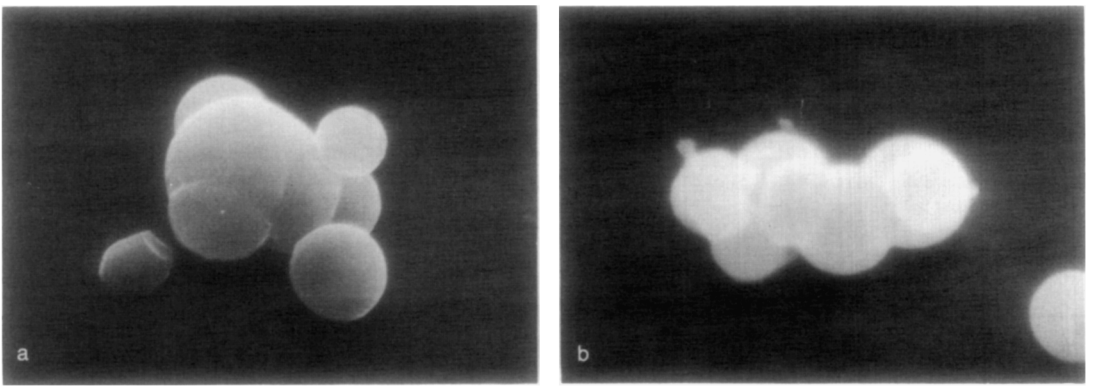

Figure 1. Scanning electron micrographs of human red cells aggregated and fused by exposure to phosphate ion and calcium. (a) After 30 min. incubation at 37°C, several stages of fusion of red cells exposed to 20 mM Ca after pre-treatment of cells with 13 mM phosphate are seen. Single cells appear at lower left and upper right, a probable doublet at lower right and a large polykaron in the center with cells in process of fusion. (b) A clump of aggregated and fusing cells show emerging vesicles. The cells of (a) and (b) were fixed after 30 min. at 37°C by 2% glutaraldehyde, dehydrated in ethanol, and critical point dried in liquid carbon dioxide. Sputter coating with gold-palladium preceded the examination of the cells in an AMR-1000 scanning microscope (X5000).

and then on incubation at 37°C they fused with increased efficiency and reduced hemolysis. Thus the visible precipitate of calcium phosphate plays little or no role in agglutination and fusion. One advantage of this method of preparation was that cell visibility under the phase microscope was much higher than under conditions where a precipitate around the cell clumps tended to obscure detail. On the other hand, if phosphate was added to a pellet of cells spun down from a calcium-saline solution, no agglutination was seen, indicating the necessity of exposing the cells to phosphate before the addition of calcium in order to achieve cell-cell bridging.

The relationships between incubation time, ATP levels, percent hemolysis, cell-associated calcium and fusion indices are shown in Fig. 2 for red cells incubated at 37°C in 20 mM calcium-saline after exposure to 44 mM phosphate. After thorough washing of freshly drawn blood in isotonic saline, one sample was suspended in 44 mM phosphate buffered saline, another sample in isotonic saline. After centrifugation, the two pellets were resuspended

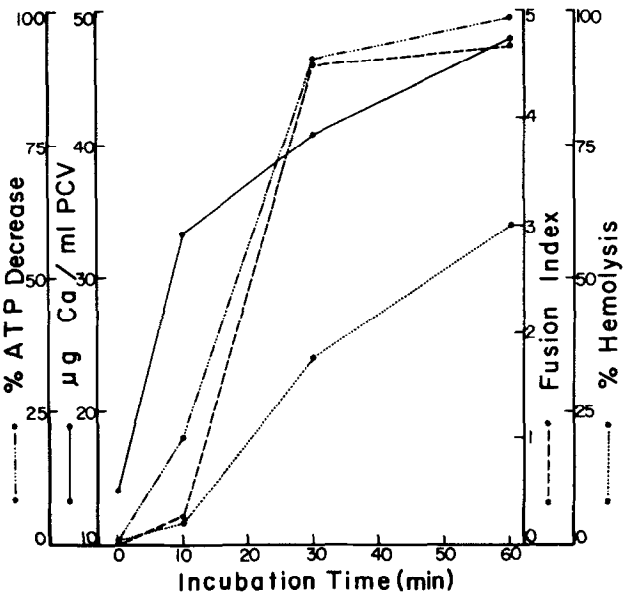

Figure 2. Effect of incubation time on ATP levels, hemolysis, cell-associated calcium and fusion indices for red cells exposed to calcium-saline containing 44 mM phosphate. Six x 1ml samples of saline-washed red blood cells were prepared at 10% PCV; four were suspended in 44 mM phosphate-buffered saline pH 7.4, and two samples in 300 mOs saline. All tubes were centrifuged at 1610 g for 5 min., following which the supernatant was withdrawn. One ml of 20 mM $CaCl_2$, buffered with 12 mM Hepes to pH 7.4 and made isotonic with NaCl, was added to each pellet and the tubes were vortexed before incubation at 37°C. At times 0, 10, 30, and 60 min. and after vortexing, 0.1 ml of suspension was removed for ATP assay (7). 20 µl of suspension was removed and diluted 50,000 x with Isoton for cell counts (Coulter Model A with 70 µm aperture). The remaining suspension in each tube was centrifuged at 2440 g. for 10 min. at 4°C and the supernatant removed for measurement of hemoglobin absorption to give percent hemolysis. The pellet of intact and fused cells and ghosts was washed (10 min. X 2445 g.) 3X in 14 ml of isotonic saline at 4°C, and prepared for calcium assay as described in Materials and Methods.

to a 10% PCV in buffered saline containing 20 mM calcium chloride. Both tubes were incubated at 37°C and aliquots removed at 0, 10, 30 and 60 min. ATP levels in the cells incubated with phosphate and calcium decreased 46 fold in 1 hour, while cell-associated calcium increased 70 fold. In cells incubated in saline plus 20 mM calcium, ATP decreased only 2 fold, while cell-associated calcium showed no change in 1 hr. Hemolysis was minimal in the saline-calcium suspension, but was 60% complete in phosphate-calcium after 1 hour of incubation.

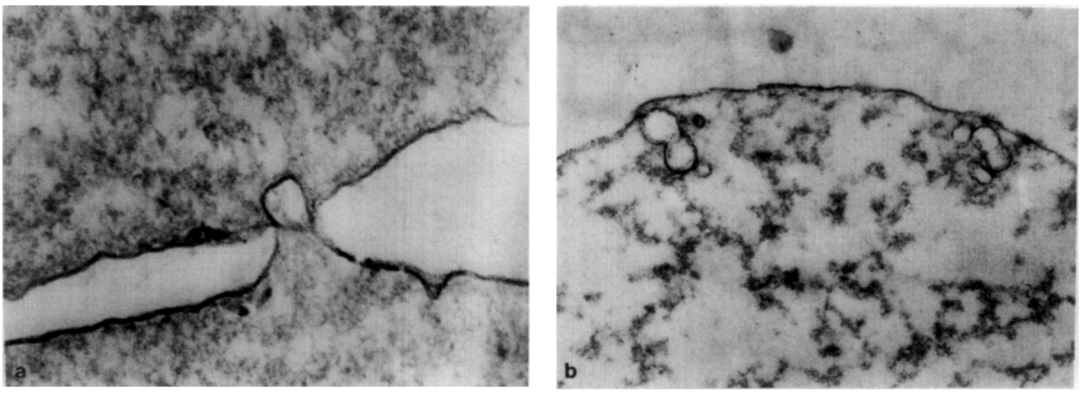

Figure 3. (a) Transmission electron micrograph of early stage of cell fusion. Calcium chloride (final concentration 20 mM), was added to a suspension (10% PCV) of red cells in 13 mM phosphate buffer. After 20 min. at 37°C the cells were fixed in 2% buffered glutaraldehyde for 30 min. followed by 2% buffered osmium tetroxide for 1 hour. Dehydration in ethanol and embedding in Epon 812 preceded the cutting of thin sections on an LKB Ultratome. Sections were stained with uranyl acetate and lead citrate. A vesicle originating from the lower cell is invaginating into the cell at top. Partial hemolysis is seen in both cells. (b) As in Fig. 3a except for an incubation time of 1 hour. Free intracellular vesicles which presumably originated in a contacting cell are seen. (a) X88,000 (b) X33,600

Entry into the red cell ghost of both calcium and phosphate has been postulated as one of the steps in fusion (10). It now appears probable that entry of phosphate into the intact red cell may be not necessary for fusion by phosphate and calcium. Red cells in saline were treated with DIDS (4,4'-diisothiocyano-2,2'-stilbene disulfonic acid; 2-10 μM), a known inhibitor of anion transport (8), for 15 minutes at 4°C and then tested for fusion. Controls established that under these conditions phosphate did not enter the cells and yet fusion efficiency was unimpaired. The entry of phosphate or calcium phosphate during hemolysis is of course not ruled out by DIDS treatment. However, it seems likely that fusion had already been initiated before hemolysis was complete so that a later entry of phosphate may have had no bearing on the fusion mechanism. Fig. 3A shows an early stage of fusion in cells containing much of their hemoglobin.

Freeze-fracture studies (5,11,12) have established that one of the common steps in fusion by various agents is an aggregation in the fusing

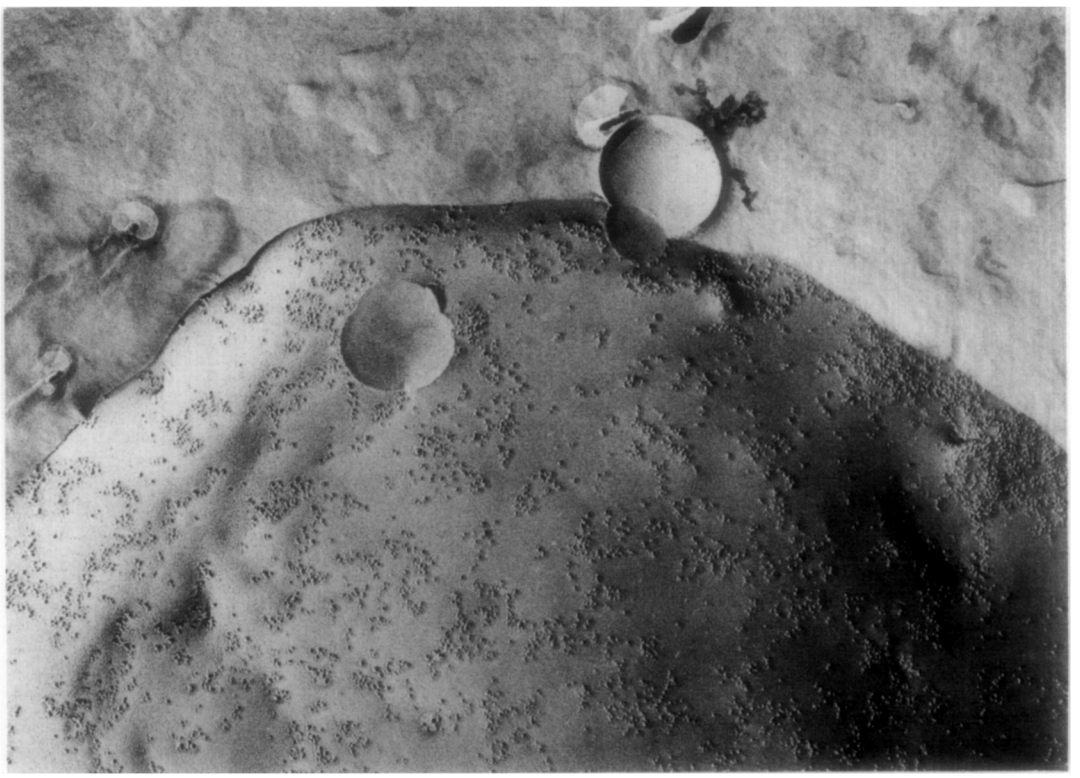

Figure 4. Aggregation of membrane-associated protein and protein-free vesiculation are seen in this replica of freeze-fractured cells which had been incubated at 37°C in 13 mM phosphate-20 mM calcium. After 20 min. incubation the suspension was fixed in 2% buffered glutataldehyde for 30 min., then washed 3X in 15 volumes of distilled water, before resuspending in 25% glycerol-water. Aliquots (2 µl) were frozen in Freon 22 at -150°C. Fracture was by the split tube method at -110°C in a Polaron freeze-etch unit. A carbon-platinum replica of the fracture surface was examined in a Philips 300 transmission electron microscope (X 53800).

membranes of membrane-associated proteins (MAP). We confirm that this is observed for fusion of intact red cells by phosphate and calcium (Fig. 4). It has been reported that intracellular calcium causes MAP aggregation and protein-free vesiculation in red cell ghosts, perhaps by interaction with spectrin (13) or by phase separation of lipid (14,15). Fig. 4 illustrates the formation of MAP-free vesicles from red cells treated with calcium and phosphate. Such vesicles may exvaginate into the medium, or as in Fig. 3A

Table I Partitioning of ATP in fusing red cells

t (min)	ATP in the pellet %	ATP in the Supernatant %	ATP Utilized %
0	100	0	0
10	68.1	7.9	24
30	52.8	8.2	39
60	32.5	5.5	62

A suspension of red cells (10% PCV) was washed in 13 mM phosphate-buffered saline, following which 27 mM $CaCl_2$ (buffered with 12 mM Hepes, pH 7.4) in saline was added. After 10 min. at room temperature, four aliquots of this suspension were incubated at 37 C for 0, 10, 30 and 60 min. The suspensions were vortexed at these time intervals and 0.1 ml removed for measurement of total ATP (7). After centrifugation at 1610 X g. for 5 min., 0.1 ml of supernatant was removed for measurement of supernatant ATP. Pellet ATP levels were obtained by subtraction of supernatant ATP from total ATP.

bud into a close neighbor at a point where fusion is being initiated. As seen in Fig. 3B these vesicles at a later stage of fusion will appear as intracellular vesicles.

In order to distinguish between a metabolic loss of ATP and a loss by hemolysis, ATP was measured in the complete fusion mixture and in the supernatant as a function of incubation time. Some typical data are shown in Table I. After 1 hour of incubation 62% of the original ATP had been utilized. Of the remaining ATP (38%), about 32% is associated with intact cells and ghosts while only 5.5% is found in the supernatant. Since hemolysis, in this experiment, was nearly complete after 1 hour it follows that most of the ATP was consumed before hemolysis. It is probable that the entry of calcium into the cell was responsible for the drop in ATP levels since the calcium pump is activated by intracellular calcium and functionally dependent on ATP. If this is true, the disparity between the ATP drop in this experi-

ment and that seen in Fig. 2 may be due to differences in intracellular calcium levels in the two experiments. The ATP still associated with the pellet of fused cells (32% of original) is worthy of note, since the amount of ATP associated with cells after hemolysis is normally much smaller.

DISCUSSION: The data presented here shows that fusion of fresh ATP-replete human red cells occurs, using calcium and phosphate ion, contrary to a previous report (6). The fusion process requires prior association of phosphate ion with the membrane since pre-treatment of red blood cells with calcium ion followed by phosphate did not result in fusion. Neither did the fusion occur when cells were added to a calcium phosphate mixture. In addition, pre-treatment of cells with DIDS, an anion transport inhibitor, did not affect the fusion efficiency suggesting that phosphate transport mediated by an anion channel may not be necessary for the fusion process.

The levels of cell-associated calcium increased by 70 fold during the incubation period with calcium and phosphate at 37 C. The consequences of such an increase in calcium content are several fold. Calcium has been shown to affect the spectrin-actin complex which presumably aggregates the membrane-associated protein (13). More over, increased levels of calcium ion causes an activation of intrinsic transglutaminase which results in membrane protein crosslinking (16). This may lead to aggregation of membrane protein. Recent studies of Alan and Mitchell (17) have shown that calcium causes an increase in the membrane content of 1,2-diacylglycerol as a result of activation of membrane-bound phosphodiesterase. They suggested that the hydrolysis of phosphatidylinositol phosphate and diphosphate from the membrane surface of erythrocytes facilitated the fusion process as a result of of removal of bulky charged groups.

The mechanism of fusion induced by calcium and phosphate appears to be different than that proposed by Alan and Mitchell (18), since in our studies pre-treatment of cells with phosphate ion is required for the fusion process induced by calcium. The formation of 1,2-diacylglycerol may occur in this

chemically induced fusion but it may not be the primary event in the fusion process. It appears that a phosphate-calcium-phosphate complex forms a bridge between adjacent erythrocytes, causing agglutination. The stability of the bilayer is reduced by an unknown mechanism leading to an increased permeability to cations and water, an increase in osmotic fragility and finally hemolysis. Calcium enters the cell at a rate which is too fast to be counteracted by the calcium pump and thus the level of ATP falls rapidly.

With accumulation of intracellular calcium aggregation of membrane protein occurs, resulting in protein-free areas of lipids. Where such areas are in close juxtaposition as a result of bridging, fusion may be initiated.

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REFERENCES:

1. Harris, H. and Watkins, J. F., Nature, Lond., 205, 640-646 (1965)
2. Ahkong, Q. F., Fisher, D., Tampion, W. and Lucy, J. A., Biochem. J., 136, 147-155 (1973)
3. Papahadjopoulos, D., Poste, G. and Schaeffer, B. E., Biochim. Biophys. Acta 323, 23-42 (1973)
4. Ahkong, Q. F., Howell, J. I., Lucy, J. A., Safwat, F., Davey, M. R. and Cocking, E. C., Nature, 255, 66-67 (1975)
5. Ahkong, Q. F., Fisher, D., Tampion, W. and Lucy, J. A., Nature, 253, 194-195 (1975)
6. Zakai, N., Kulka, R. G. and Loyter, A., Nature, 263, 696-699 (1976)
7. Stanley, P. E. and Williams, S. G., Analytical Biochem. 29, 381-392 (1969)
8. Cabantchik, Z. I. and Rothstein, A., J. Memb. Biol. 15, 207-226 (1974)
9. Okada, Y. and Tadokoro, J., Ex. Cell. Res., 26, 108-118 (1962)
10. Zakai, N., Kulka, R. G. and Loyter, A., Proc. Nat. Acad. Sci., 74, 2417-2421 (1977)
11. Bachi, T., Aguet, M. and Howe, C., J. Virol.,11, 1004-1012 (1973)
12. Vos, J., Ahkong, Q. F., Botham, G. M., Qurik, S. D. and Lucy, J. A., Biochem. J., 158, 651-653 (1976)
13. Elgsaeter, A., Shotton, D. M. and Branton, D., Biochim. Biophys. Acta, 426, 101-122 (1976)
14. James, R. and Branton, D., Biochim. Biophys. Acta, 323, 378-390 (1973)
15. Jacobson, K. and Papahadjopoulos, D., Biochem., 14, 152-161 (1975)
16. Lorand, L., Weissman, L. B., Epel, D. L. and Lorand, J. B., Proc. Natl. Acad. Sci. 73, 4479-4481 (1976)
17. Allan, D. and Mitchell, R. H., Biochim. Biophys. Acta 508, 277-286 (1978)