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Divalent cations, phospholipid asymmetry and osmotic swelling in electrically-induced lysis, cell fusion and giant cell formation with human erythrocytes

Lin Ye Song, Quet F. Ahkong [1], Jocelyn M. Baldwin, Rita O'Reilly and Jack A. Lucy

Department of Biochemistry and Chemistry, Royal Free Hospital, School of Medicine, University of London, London (UK)

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We have previously reported that acidic phospholipids are exposed at the surface of human erythrocytes when the cells are subjected to electrical breakdown. It has now been shown that the prothrombinase assay, which was used previously for the determination of acidic phospholipids, is specific for phosphatidylserine under the conditions of our experiments. In the light of this finding, we have investigated and characterised factors that govern cell lysis, cell fusion, and the formation of giant cells induced by electrical breakdown with human erythrocytes in media of low ionic strength. Divalent cations (1.1 mM) protected the cells against haemolysis, in the order $Mn^{2+} > Ca^{2+} > Ba^{2+} > Mg^{2+} \gg Zn^{2+}$, whereas about 99% of the cells lysed immediately on breakdown in the presence of Na^+ or K^+ (2.1 mM), or Al^{3+} (0.95 mM). The lengths of pearl chains of fused erythrocytes formed was similarly greatest with Mn^{2+} and decreased progressively with Ba^{2+}, Zn^{2+}, Ca^{2+} and Mg^{2+}. No cell fusion occurred with Na^+, K^+, or Al^{3+}. It is suggested that interactions with phosphatidylserine, which is exposed at the cell surface by electrical breakdown, may enable Mn^{2+}, Ba^{2+} and Ca^{2+} ions to inhibit cell lysis (via membrane resealing) and facilitate cell fusion. Following electrically-induced cell fusion, erythrocytes round-up into giant cells. It has previously been proposed that Ca^{2+} ions accelerate the rounding-up process. However, data are presented which show that, as with erythrocytes treated with Sendai virus, the formation of rounded, giant cells following cell fusion depends on the osmotic swelling properties of permeabilised erythrocytes. Osmotic swelling may also have induced any hemi-fused cells present to fuse completely. Zn^{2+} ions anomalously enabled erythrocytes to round-up very rapidly into giant cells following electrical breakdown. This phenomenon may result from an interaction of Zn^{2+} ions with cysteine groups in membrane proteins, which decreases the immediate loss of ions that occurs when erythrocytes are subjected to electrical breakdown in low-ionic-strength media.

Introduction

In early work on the fusion of erythrocytes by the Sendai virus, Mn^{2+} virtually prevented haemolysis induced by the virus and permitted a high fusion index to be obtained [1]. It was, therefore, suggested that bivalent metal cations facilitate cell fusion by inhibiting cell lysis. More recently it was observed that, when monolayers of human erythrocytes were induced to swell by the entry of small non-electrolytes, lysis occurred without cell fusion in the absence of Ca^{2+}, whereas in the presence of Ca^{2+} many of the swelling cells fused before they lysed [2,3]. We now report that Mn^{2+}, Ca^{2+} and Ba^{2+} similarly inhibit cell lysis and facilitate cell fusion with human erythrocytes that are subjected to electrical breakdown in media of low ionic strength. In the light of new data on the specificity of the prothrombinase assay for phosphatidylserine, it is suggested that interactions with phosphatidylserine that is exposed at the cell surface may enable Mn^{2+}, Ca^{2+} and Ba^{2+} to inhibit lysis and facilitate cell fusion, at least with erythrocytes that are subjected to electrical breakdown.

Although cell fusion is initiated within microseconds of electrical breakdown, the time taken to achieve the final spherical shape was found to vary in early work from seconds to 60 min, depending on the species of cell, as well as on the number of cells being fused. It was proposed that the time needed for complete fusion in differing cell types depends on their membrane fluidities, and on the properties of the cytoskeleton

Correspondence to: J.A. Lucy, Department of Biochemistry and Chemistry, Royal Free Hospital School of Medicine, Rowland Hill Street, London, NW3 2PF, UK.

[1] Present address: Skin Barrier Research Laboratory, Department of Medicine, University of British Columbia, 2211 Wesbrook Mall, Vancouver, British Columbia, Canada V6T 2B5.

within the cells [4,5]. In many instances, and particularly in the case of fused mammalian cells, the speed with which the spherical shape was assumed appeared to depend on the presence of ions, with 1 mM Ca^{2+} being especially effective. By contrast, no evidence was found for the involvement of osmotic processes in the rounding-up of fused cells [4]. Relatively little work has since been done on the factors that affect the morphology of intact cells after electrically-induced cell fusion, although it has recently been concluded that the spectrin network and a non-osmotic force controls the morphology of erythrocyte ghosts fused by electrical breakdown [6]. We now report observations which indicate that osmotic swelling, which results from the entry into cells of sugar molecules via electropores in the plasma membrane, is actually responsible for the rounding-up of human erythrocytes into giant cells, following exposure to electrical breakdown pulses in low ionic strength media. It is suggested that, as with erythrocytes treated with Sendai virions, osmotic swelling appears to be the driving force that results in permeabilised cells, in which membrane fusion sites are present, being able to expand into giant cells.

We have also found that, in the presence of Zn^{2+} ions, erythrocytes do not shrink on electrical breakdown in low ionic strength media, and that they subsequently swell very rapidly into giant cells. This may result from an interaction of Zn^{2+} ions with cysteine groups in membrane proteins that prevents the loss of ions that would otherwise occur on electrical breakdown.

Materials and Methods

Materials

Bovine blood coagulation factors (factor Xa, prothrombin, thrombin) were from Sigma. Factor V was from Diagnostic Reagents and was activated before use as described in Ref. 3. The chromogenic substrate for thrombin, S2238, (*H*-D-phenylalanalyl-L-pipecolyl-L-arginine-*p*-nitroanilide dihydrochloride) was from KabiVitrum. Phosphatidylcholine (type XI-E from egg yolk), dioleoylphosphatidylethanolamine, phosphatidylserine (from bovine brain) and inulin (M_r 5000) were from Sigma. 6-Carboxyfluorescein diacetate was from Molecular Probes.

Methods

Assay of procoagulant activity. Solutions of phospholipids were prepared in chloroform/methanol (1:1 (v/v)) and were mixed (by weight) in the following ratios: phosphatidylcholine/phosphatidylserine (4:1), phosphatidylcholine/phosphatidylethanolamine (4:1), phosphatidylcholine/phosphatidylserine (9:1), phosphatidylcholine/phosphatidylserine/phosphatidylethanolamine (8:1:1) and phosphatidylcholine (100%), The mixed solutions were taken to dryness under N_2 and the dried phospholipids were suspended, at a concentration of 0.5 mg/ml, in 40 mM Hepes buffer at pH 7.4 containing 110 mM NaCl. They were then sonicated for 10 min on ice. Suspensions of the phospholipid vesicles were diluted 200-fold into an aqueous solution of 250 mM sucrose, 10 mM histidine, 1.1 mM $CaCl_2$ and 100 μl aliquots were assayed for their procoagulant activity exactly as described in Ref. 7.

Exposure of erythrocytes to electrical breakdown pulses. Human erythrocytes were washed and freed from leucocytes as described in Ref. 8, and resuspended in a buffered salt solution (124 mM NaCl, 40 mM Hepes buffer at pH 7.4). 5 μl of a packed suspension of the cells was added to 950 μl of a solution of sucrose or other sugar (250 mM unless otherwise stated), containing 1.1 mM of a divalent cation (Ca^{2+} unless otherwise stated) and 10 mM histidine, which had a final conductivity of 235 μS/cm. Unless otherwise specified, a stainless steel pipetting annular electrode chamber (Kruss) with an electrode gap of 500 μm was used with a Zimmermann Cell Fusion System (GCA). 1.5 min after adding the packed cells to the buffered fusion medium, the erythrocytes were aligned by dielectrophoresis in an AC field of 0.16 kV/cm at 1.5 MHz. After a further 0.5 min, three, square-wave, 99-μs electric field pulses (5 kV/cm) were applied to the cells at 1-s intervals, and the AC field was removed 0.5 min later. The pulsed cell sample was removed from the electrode chamber, and portions used to determine the cell fusion index, to investigate the number of cells in fused chains of erythrocytes, to determine cell lysis, and to investigate cell swelling, cell rounding, and the formation of giant cells. All experiments were done at 25°C.

In experiments on Ca^{2+} ions, osmotic swelling, and the formation of giant cells, erythrocytes were aligned by dielectrophoresis at a comparatively high field strength (0.4 kV/cm at 1.5 MHz) between two electrodes that were 200 μm apart (using a function generator, type TE7702, Toellner Electronic, M. Werner); the subsequent electrical breakdown pulse was applied from a pulse generator (type 214B, Hewlett-Packard) [9]. This AC field induced very close contact of the aligned erythrocytes, and enabled them to be fused in Ca^{2+}-free media of low conductivity by a single DC field pulse (20 μs) of only 3.5 kV/cm.

Cell lysis. Samples of pulsed cells were maintained at 25°C for 23 min, and then centrifuged at 2000 × *g* for 3 min. Cell lysis was estimated from the absorbance at 405 nm of the haemoglobin in the supernatant by comparing the absorbance values obtained with those from a preparation of cells which had been totally lysed, by adding 5 μl of packed cells to 950 μl of distilled water, and subsequently sonicated.

Cell fusion. Erythrocytes were labelled with 6-carboxyfluorescein as previously described [10], but the labelled cells were then mixed with unlabelled cells in the proportion of 1:4.5. Exposure of human erythrocytes to an electrical breakdown voltage is followed by the almost-instantaneous diffusion of 6-carboxyfluorescein from labelled to unlabelled cells [9]. The percentage of cell fusion was determined using a Nikon Diaphot-TMD microscope, fitted with a ×100 oil immersion objective, an HBO 100 W mercury lamp and a B filter cassette to view the carboxyfluorescein probe by epi-fluorescence. Cells were counted on a microscope slide in randomly selected fields after their exposure to breakdown pulses. Between 100 and 300 cells (depending on the incidence of fusion) were counted in triplicate for each experimental condition. The percentage cell fusion was defined as $B/A(\times 100)$, where B was the number of fused fluorescent cells in a pearl chain of erythrocytes and A was the total number of cells counted. Fluorescent cells which were adjacent to unlabelled cells in pearl chains were not included in B. All cells in such chains were, however, included in A. Single cells (labelled and unlabelled) were also included in A. Pearl chains which had no fluorescent cells were not counted because the presence of cytoplasmic connections in such chains cannot be established or excluded on a quantitative basis.

The mean number of cells in the pearl chains formed when (unlabelled) erythrocytes were subjected to electric breakdown pulses was calculated according to the following formula:

$$\big((M^2\cdot\alpha)+\big((M-1)^2\cdot\beta\big)+..\text{ etc..}+2^2\cdot(\text{No. of 2-cell chains})$$
$$+(\text{No. of single cells})\big)/\text{total number of cells},$$

where M was the number of cells in the longest chain, α was the number of cell chains containing M cells and β was the number of cell chains containing $(M-1)$ cells. Between 200 and 400 cells were counted for each sample except that, because of the high percentage of cell lysis with erythrocytes that were subjected to electrical breakdown in the presence of Zn^{2+}, only 110, 167 and 183 cells were counted in the replicate experiments with this cation. Photographs of pearl chains of cells, and of the transformation of fused cells into giant cells, were obtained with a Nikon Diaphot-TMD microscope, fitted with a ×100 oil immersion objective, using Technical Pan (Kodak) and Pan F (Ilford) films.

Results

Specificity of prothrombinase assay for phosphatidylserine

We have previously reported that acidic phospholipids are exposed at the surface of human erythrocytes when the cells are subjected to electrical breakdown [7]. In that work, the rate of conversion of prothrombin to thrombin by the enzyme complex (factor Xa-factor Va) was used as a convenient, sensitive, and semi-quantitative way of monitoring the surface exposure of acidic phospholipids. This method had earlier been employed by other workers to monitor the surface exposure of phosphatidylserine in the plasma membranes of erythrocytes [11] and platelets [12], but it was subsequently reported that the assay detects phosphatidylethanolamine almost as well as phosphatidylserine in mixtures with phosphatidylcholine [13].

TABLE I

Specificity of prothrombinase assay for phosphatidylserine

The percentage prothrombinase activity was determined as described in Materials and Methods for phospholipid vesicles that were suspended, at a final concentration of 2.5 μg/ml, in a solution of 250 mM sucrose containing 10 mM histidine and 1.1 mM $CaCl_2$.

Phospholipid composition	Prothrombinase activity (nM thrombin/min)
PC	0.025
PC:PE (4:1)	0.043
PC:PS (9:1)	61.2
PC:PS:PE (8:1:1)	74.8
PC:PS (4:1)	127.3

The specificity of the prothrombinase assay has now been further investigated with phospholipid vesicles. As the prothrombinase activity can be greatly affected by the reaction conditions, phospholipid vesicles were assayed under the conditions (in 250 mM sucrose solution containing 10 mM histidine) that were employed previously with erythrocytes subjected to electrical breakdown [7]. Bovine brain phosphatidylserine was many orders of magnitude more effective in catalysing the conversion of prothrombin to thrombin than either dioleoylphosphatidylethanolamine or egg phosphatidylcholine (Table I). Phosphatidylethanolamine appeared to be marginally more active in the presence of phosphatidylserine than in its absence. It thus appears that the enhanced procoagulant activity previously observed with human erythrocytes after electrical breakdown [7] is specifically attributable to exposure of phosphatidylserine at the cell surface. We have now investigated and characterised factors that govern cell lysis, cell fusion and the formation of giant cells with human erythrocytes subjected to electrical breakdown, in the light of this conclusion.

Effect of cations on cell lysis induced by electrical breakdown

Human erythrocytes (aligned by dielectrophoresis in a solution of 250 mM sucrose and 10 mM histidine) were subjected to three electrical breakdown pulses of

99 μs (5 kV/cm) at 1-s intervals in the presence of 1.1 mM concentrations of different divalent cations (final conductivity 235 μS/cm). Table II shows that the ability of the cations to protect the cells against haemolysis by electrical breakdown decreased progressively in the order $Mn^{2+} > Ca^{2+} > Ba^{2+} > Mg^{2+} \gg Zn^{2+}$. With 250 mM sucrose solutions of the same conductivity (235 μS/cm), but which contained Na^+, K^+, (2.1 mM) or Al^{3+} (0.95 mM) ions, about 99% of the cells lysed immediately on electrical breakdown.

Ca^{2+} ions are known to stimulate the resealing of lysed human erythrocytes by stimulating the contraction of osmotically-induced holes in the plasma membrane. In this respect, the potency of Ca^{2+} was found to exceed that of Mg^{2+}, which greatly surpassed Na^+, and it was suggested that Ca^{2+} facilitated the resealing of osmotically-lysed erythrocytes by stoichiometric binding to (unspecified) anionic groups on the membrane [14]. It, therefore, seems possible that, in our experiments, Ca^{2+} ions facilitated membrane resealing in electrically-pulsed erythrocytes by binding to molecules of phosphatidylserine that were exposed on the cell surface by electrical breakdown.

Effect of cations on cell fusion induced by electrical breakdown

The number of fused cells in the pearl chains, which were formed when human erythrocytes were exposed to breakdown pulses as above, was greatest in the presence of Mn^{2+}, and decreased progressively with Ba^{2+}, Ca^{2+} and Mg^{2+} (Table III). It is also apparent from Table III that this decreasing effectiveness is inversely related to previously reported data on (i) the threshold concentrations of cations required to induce the fusion of 500-Å diameter vesicles of phosphatidylserine; (ii) the concentrations of cations needed to increase the interfacial tension in monolayer membranes of phosphatidylserine by 6–8 dyne/cm [15].

TABLE II

Effects of divalent cations on the lysis of human erythrocytes subjected to electrical breakdown pulses

Human erythrocytes, aligned by dielectrophoresis in a 250 mM solution of sucrose, containing 10 mM histidine and 1.1 mM concentrations of different divalent cations, were subjected to three electrical breakdown pulses of 99 μs (5 kV/cm) at one second intervals. The percentage haemolysis was determined, for the numbers of separate experiments shown in brackets, as described in Materials and Methods.

Divalent cation	% cell lysis ± S.D.
Mn^{2+}	14.4 ± 1.4 (3)
Ca^{2+}	15.1 ± 0.6 (4)
Ba^{2+}	21.5 ± 2.9 (3)
Mg^{2+}	24.9 ± 3.2 (4)
Zn^{2+}	44.1 ± 2.4 (3)

TABLE III

Effects of divalent cations on the fusion of human erythrocytes induced by electrical breakdown, and on the properties of model membrane systems of phosphatidylserine

Human erythrocytes, aligned by dielectrophoresis in a solution of 250 mM sucrose and 10 mM histidine, were subjected to three electrical breakdown pulses of 99 μs (5 kV/cm) at 1-s intervals in the presence of 1.1 mM concentrations of five different divalent cations. The mean number of cells in the chains of fused cells formed was determined, for the numbers of separate experiments shown in brackets, as described in Materials and Methods. The threshold concentrations of divalent cations for the fusion of 500 Å diameter vesicles of phosphatidylserine were derived by interpolation from Fig. 2 in Ref. 15. The concentrations of cations found to increase the interfacial tension of monolayers of phosphatidylserine by 7–8 dyne/cm are from Table I in Ref. 15.

Divalent cation	No. of cells in chains of electrofused cells with 1.1 mM cation ± S.D.	Threshold concentration for the fusion vesicles of phosphatidylserine (mM)	Concentration for increase in tension of 7–8 dyne cm^{-1} in monolayers of phosphatidylserine (mM)
Mn^{2+}	4.48 ± 0.28 (4)	0.85	0.7
Ba^{2+}	4.26 ± 0.22 (3)	–	0.8
Zn^{2+}	3.72 ± 0.14 (3)	–	–
Ca^{2+}	3.50 ± 0.07 (3)	1.3	1.0
Mg^{2+}	2.28 ± 0.04 (3)	6.0	6.0

Our observations therefore support the concept that the surface exposure of acidic phospholipids plays an important role in the fusion of human erythrocytes induced by electrical breakdown.

Erythrocytes that were subjected to electrical breakdown in 250 mM sucrose solutions which contained Na^+, K^+ (2.1 mM) or Al^{3+} (0.95 mM) ions (conductivity 235 μS/cm) were unable to form pearl chains. In this connection it is relevant that up to 1 M concentrations of Na^+ and K^+ failed to induce the fusion of small unilamellar vesicles of phosphatidylserine, or to increase the interfacial tension in monolayer membranes of phosphatidylserine [15].

The data presented here show that, apart from the behaviour of erythrocytes with Zn^{2+}, there is a general relationship between protection against haemolysis by cations and the formation of chains of fused cells in the presence of these ions. Thus, Mn^{2+} was most effective in protecting the cells against electrically-induced lysis and in facilitating the formation of chains of erythrocytes, while Mg^{2+} was the least effective on both counts. Ca^{2+} and Ba^{2+} were intermediate between Mn^{2+} and Mg^{2+}, although not in the same order. Na^+, K^+ and Al^{3+}, gave virtually no protection against haemolysis, and the cells were not fused by electrical breakdown pulses.

Zn^{2+} has been reported to induce the fusion of vesicles composed of dipalmitoylphosphatidylcholine/phosphatidylserine (50%/50% or 60%/40%) at con-

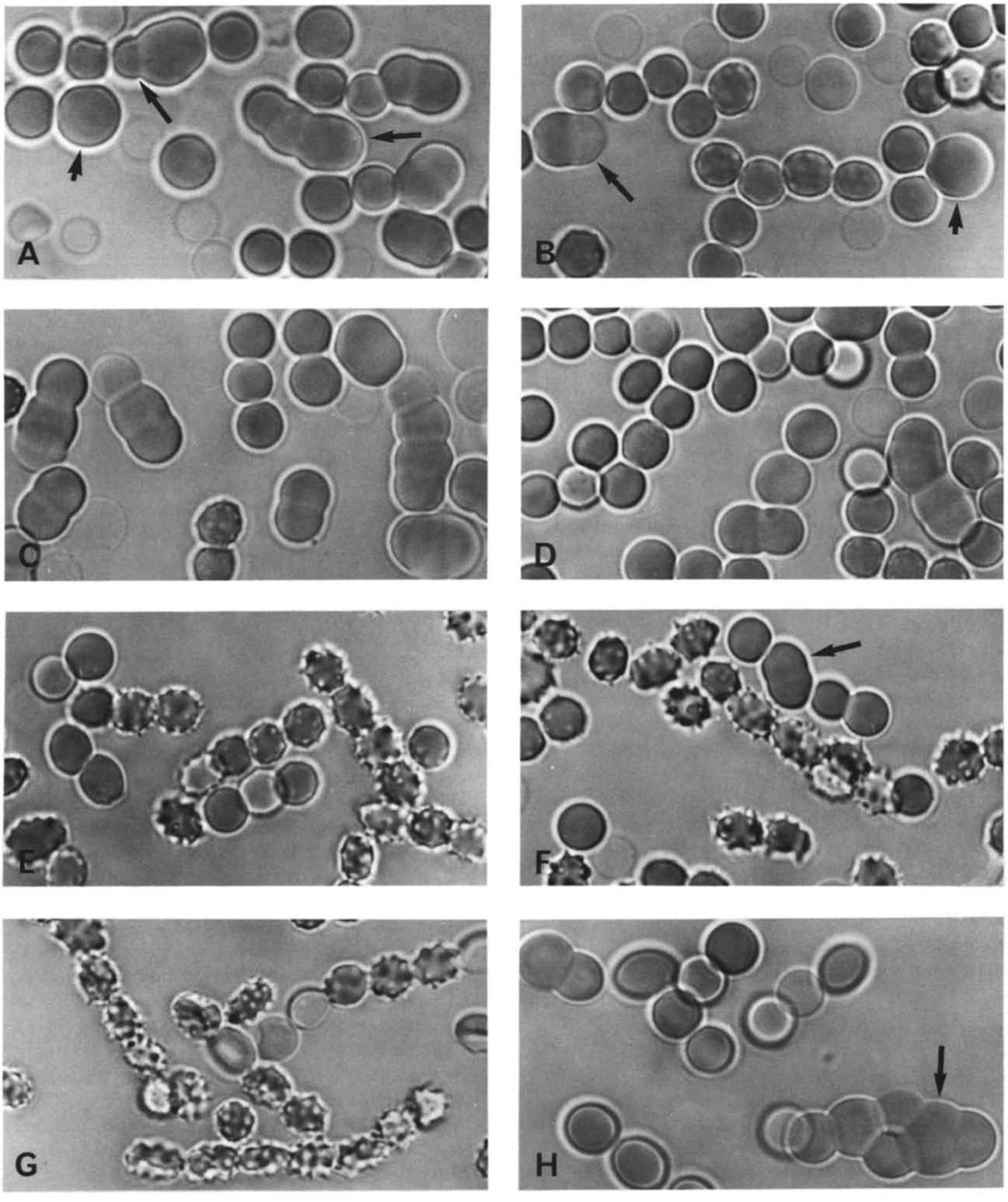

Fig. 1. Panels A–G show human erythrocytes at 25°C, aligned by dielectrophoresis in 250 mM solutions of sugars (containing 10 mM histidine and 1.1 mM Ca^{2+}), that were subjected to three electrical breakdown pulses of 99 μs (5 kV/cm) at 1-s intervals. Panel A (with erythritol, 45 min after breakdown): most of the cells were swollen, fused cells were rounding-up into giant cells (long arrows) and some spherical giant cells (short arrow) were present. Panel B (60 min with ribitol), fused cells were rounding into giant cells (long arrow), spherical giant cells were present (short arrow), but many cells exhibited evidence of shrinkage. Panel C (90 min with ribitol), the cells were comparable to 45 min preparations with erythritol. Panel D (90 min with mannitol), the rounding-up process was less advanced than after 90 min with ribitol. Panel E (2 h with sucrose) shows persistent cell shrinkage. Panel F (5 h with sucrose) shows occasional giant cell formation (arrow) among the shrunken cells. Panel G shows that cell swelling, rounding-up and giant cell formation were markedly inhibited after 60 min by the presence of 30 mM dextran (M_r 4000–6000) in the erythritol medium. Panel H shows erythrocytes, subjected to electrical breakdown as for panels A–G, except that 1.1 mM Zn^{2+} was present instead of 1.1 mM Ca^{2+}. Extensive cell rounding occurred, and a giant cell was present (arrow) only 15 min after breakdown in sucrose media that contained Zn^{2+} ions. Magnification: 460×. Each experiment was done at least three times, and the micrographs shown are representative of the observations made.

siderably lower concentrations than Ca^{2+} [16]. It might therefore be expected that, as with Mn^{2+}, relatively long chains of fused cells would be formed by electrical breakdown in the presence of Zn^{2+}. However, replacement of 1.1 mM Ca^{2+} in the fusion medium by 1.1 mM Zn^{2+} had little effect on the length of the chains of fused cells. As discussed below, the swelling behaviour of erythrocytes subjected to electrical breakdown in the presence of Zn^{2+} was also found to be anomalous.

Ca^{2+} ions, osmotic swelling and the formation of giant cells

In early work, it was reported that the addition of a medium containing 1 mM Ca^{2+} ions (but otherwise of low conductivity) was often required about 1 min after the breakdown pulse, or else the cells did not become spherical [17]. However, we obtained a quite different result when erythrocytes, aligned by dielectrophoresis (0.4 kV/cm at 1.5 MHz) were subjected to a single electrical breakdown pulse of 20 μs (3.5 kV/cm) in hypotonic solutions of 150–200 mM erythritol (containing 5 mM histidine) without either Ca^{2+} or commercial pronase (which stabilises cells against the AC field and DC pulses at least partly because it contains Ca^{2+} [18,19]). (The comparatively high AC field strength used to align the cells by dielectrophoresis was applied as described in Materials and Methods.) Under these conditions, the fused cells swelled rapidly, giant cells formed in 10–30 s and cell lysis was complete within a few minutes. In a separate experiment, the addition of small quantities of distilled water to erythrocytes after their exposure to breakdown pulses in a sucrose-containing medium caused them to round-up into giant cells. These findings indicated that cell swelling, rather than Ca^{2+} ions, facilitates the rounding-up of electrically-fused erythrocytes into giant cells.

Erythrocytes lose ions and shrink immediately following electrical breakdown. The permeabilised cells than swell osmotically because of their impermeable haemoglobin, and the rate of swelling is decreased by the presence of molecules of increasing size [20]. In order to investigate further the role of osmotic swelling in the rounding-up of fused erythrocytes into giant cells, we therefore subjected them to electrical breakdown in solutions of carbohydrate molecules of increasing size (which also contained 1.1 mM Ca^{2+}). Sugars ranging from the 4-carbon sugar, erythritol (radius approx. 3.1 Å), to the 12-carbon sugar, sucrose (radius approx. 4.4 Å) were investigated [21]. 5 min after the pulses, cells in 250 mM erythritol media were much less shrunken than cells in 250 mM sucrose media. After 45 min with erythritol, the cells were extensively swollen, a high proportion of the fused cells were rounding-up into giant cells, and some spherical giant cells were present (Fig. 1A). By comparison, the rounding-up was considerably less advanced after 60 min withathe 5-carbon sugar, ribitol (adonitol), and evidence of cell shrinkage was still apparent (Fig. 1B). After 90 min, cells with ribitol (Fig. 1C) were comparable with the 45 min preparations of cells with erythritol (Fig. 1A), and the rounding-up was more advanced than in 90 min preparations with the 6-carbon sugar, mannitol (Fig. 1D). Cells in 250 mM sucrose solutions rounded-up extremely slowly, and most of the cells remained shrunken after 2 h (Fig. 1E). Occasional giant cells were observed 5 h after breakdown, but shrunken erythrocytes were in a majority (Fig. 1F).

When a non-permeable dextran (M_r 4000–6000, 30 mM) was present in the 250 mM erythritol medium, cell swelling, rounding-up and giant cell formation were markedly inhibited by comparison with cells in erythritol medium (280 mM) without dextran. After 60 min with added dextran, the erythrocytes were very shrunken, and no cell rounding or giant-cell formation was observed (Fig. 1G). Similarly, cells in 250 mM erythritol media which contained 30 mM poly(ethylene glycol) 3350, or 3 mM inulin (M_r 5000), rounded-up more slowly than cells in the presence of erythritol alone. If these poly(ethylene glycol)- or inulin-containing media were subsequently replaced by a medium containing erythritol alone, cell swelling and giant cell formation proceeded unhindered. These several observations showed that the rounding-up of fused human erythrocytes into giant cells depends on osmotic swelling and parallels the well-documented swelling properties of erythrocytes which have been permeabilised by electrical breakdown.

Osmotic swelling, hemi-fusion and cell fusion

With relatively short breakdown pulses (20 μs) and low applied DC voltages (2.7 kV/cm), human erythrocytes exhibit hemi-fusion, i.e., membrane fusion without cytoplasmic fusion [22]. However, in the present experiments, the relatively long pulses (99 μs) and high breakdown voltage (5 kV/cm) would have resulted in

TABLE IV

Effects of sugars on the cytoplasmic fusion of human erythrocytes induced by electrical breakdown pulses

Human erythrocytes, aligned by dielectrophoresis in 250 mM solutions of different sugars, containing 10 mM histidine and 1.1 mM Ca^{2+}, were subjected to three electrical breakdown pulses of 99 μs (5 kV/cm) at 1-s intervals. The percentage of cell fusion was determined, for the numbers of separate experiments shown in brackets, as described in Materials and Methods.

Non-ionic solute	%cell fusion ± S.D.
Erythritol	57.9 ± 1.7 (3)
Ribitol	53.6 ± 3.5 (3)
Mannitol	53.1 ± 2.9 (3)
Sucrose	50.8 ± 2.8 (3)

the majority of the pulsed cells being completely fused. Table IV shows that the percentage cell fusion for erythrocytes in 250 mM solutions of different sugars decreased only slightly in the following order: erythritol > ribitol (adonitol) ≧ mannitol > sucrose. It would therefore seem that cell fusion, unlike the formation of giant cells, is not markedly affected by the size of the sugar present. The slightly higher percentage cell fusion observed with erythritol, by comparison with the other three sugars (Table IV), may nevertheless have resulted from osmotic swelling in erythritol media that induced any hemi-fused cells present to fuse completely. This interpretation is supported by an earlier finding that delayed cell fusion occurred when electrically-permeabilised erythrocytes were allowed to swell in 200 mM erythritol [23]. Hemi-fused human erythrocytes, produced by electrical breakdown, also gave rise to completely fused cells on heating to about 50°C [22].

Zn^{2+} ions and the formation of giant cells

Zn^{2+} apparently inhibited the immediate loss of ions that occurred when erythrocytes were subjected to electrical breakdown in low-ionic-strength media, since the cells failed to shrink immediately after breakdown in the presence of 1.1 mM Zn^{2+} and they subsequently swelled very rapidly. Thus, large numbers of giant cells were present after only 10–15 min (Fig. 1H). This rapid cell swelling was consistent with the high level of lysis observed with erythrocytes subjected to breakdown in the presence of Zn^{2+} (Table II). By contrast, the addition of Zn^{2+} to a Ca^{2+}-containing medium after electrical breakdown did not accelerate cell swelling and the formation of giant cells, indicating that Zn^{2+} did not increase the size of the pores through which sucrose enters electropermeabilised erythrocytes.

Cadmium and cobalt ions had similar effects to Zn^{2+}, but attempts to mimic the action of Zn^{2+} with 1 mM concentrations of inhibitors of voltage-dependent K^+ channels (4-aminopyridine and tetraethylammonium chloride) were unsuccessful.

Discussion

Although it has been reported that the prothrombinase assay detects phosphatidylethanolamine almost as well as phosphatidylserine in mixtures with phosphatidylserine [13], the assay was highly-specific for phosphatidylserine under the conditions of our experiments. Consequently, we conclude that the enhanced procoagulant activity of human erythrocytes after electrical breakdown [7] is due to a movement of phosphatidylserine from the inner to the outer leaflet of the plasma membrane. In the present work, Mn^{2+} was most effective and Mg^{2+} least effective in facilitating the formation of pearl chains of fused cells on electrical breakdown. Ca^{2+}, although less effective than Mn^{2+}, was far more effective than Mg^{2+}. These relationships are consistent with the idea that exposure of phosphatidylserine at the cell surface plays an important role in electrically-induced cell fusion, since these three ions have comparable activities in the fusion of vesicles of phosphatidylserine (Table III) [15].

In our experiments Mn^{2+} was also most effective, and Mg^{2+} least effective, in protecting the cells against lysis resulting from electrical breakdown in low-ionic-strength media. Again, Ca^{2+} was less effective than Mn^{2+}, but far more effective than Mg^{2+}. So far, no molecular explanation appears to have been put forward for the ability of Mn^{2+} and Ca^{2+} to facilitate cell fusion and inhibit lysis simultaneously [1–3]. We propose that this relationship results from the ability of these cations to interact with phosphatidylserine, at least with erythrocytes that are induced to fuse by osmotic swelling, or by electrical breakdown. Thus the ability of Ca^{2+} to form a bridge between phosphatidylserine that is exposed on the surfaces of adjacent erythrocytes [3,7] will facilitate the close approach of closely apposed membranes that is necessary for cell fusion. Since Ca^{2+} is thought to facilitate the resealing of lysed human erythrocytes by stoichiometric binding to anionic groups on the membrane [14], we suggest that Ca^{2+} additionally interacts with phosphatidylserine at the cell surface to facilitate membrane resealing following electrical breakdown, thus stabilising the cells and allowing them to fuse. (Ca^{2+} ions that enter the permeabilised cells may similarly facilitate resealing by binding to phosphatidylserine on the cytoplasmic surface.)

It is relevant that Papahadjopoulos [24] showed that monolayers of phosphatidylserine at an air-water interface interact strongly with low concentrations of divalent cations (10^{-4} to 10^{-3} M) in the presence of physiological concentrations of univalent salts, and that the interactions are accompanied by a decrease in surface pressure (condensation). He suggested a structure for the complex of phosphatidylserine with Ca^{2+}, in which each Ca^{2+} ion interacts (via six coordination bonds) with four phospholipid molecules to give a linear, polymeric arrangement.

Following electrically-induced cell fusion, erythrocytes round-up into giant cells. It has previously been proposed that Ca^{2+} ions, rather than osmotic forces, accelerate the rounding-up of cells after fusion has been induced by electrical breakdown [4]. By contrast, the importance of colloid osmotic swelling in the behaviour of human erythrocytes, following their permeabilisation by electrical breakdown, has been well-documented [20,21,25–27]. Thus, DC pulses of approx. 4 kV/cm for 5–120 μs give rise to defects in the plasma membrane, which have the properties of aqueous holes with definable radii and selectivities, and which permit the passage of ions and small molecules but not haemoglobin. The permeabilised cells swell

osmotically because of their impermeant haemoglobin, and they lyse when the cell volume approaches 155% of the normal value. The rates of swelling and of haemolysis are decreased by the presence of molecules of increasing size. Added oligosaccharides or small proteins, which are larger than the size of the electropores, retard haemolysis indefinitely because they osmotically balance the impermeant haemoglobin in the cytoplasm [21]. At 37°C, resealing of the pores occurs within minutes to hours. Thus, for pores that permit permeation of Rb^+ but not sucrose, resealing takes about 20 min. Pores that admit sucrose initially, as in our experiments, need about 20 h to reseal completely [25].

It is clear from our observations on the electrical breakdown of erythrocytes in the presence of variously permeable sugars that the rounding-up of the electrofused cells into giant cells parallels the swelling of individual erythrocytes. Thus, immediately following exposure to breakdown pulses, human erythrocytes rapidly lose ions and shrink [20]. Erythritol can enter the pulsed cells, via the electropores, more rapidly than sucrose [21]. This allows the formation of giant cells, and lysis of the electrofused cells, to occur more rapidly in the presence of erythritol than in the presence of sucrose. However, electropores that are sufficiently large to admit sucrose remain open for many hours. As a result, some fused erythrocytes in a sucrose-containing medium round-up in 5 h. The relationship between the rounding-up of electrofused cells, giant cell formation, and the swelling of individual electropermeabilised cells, is further demonstrated by the fact that the presence of large impermeable molecules in the medium inhibited cell rounding and the formation of giant cells.

Our observations on the role of cell swelling in the formation of giant cells following electrical breakdown also parallel the well-established importance of osmotically-induced cell swelling in the formation of giant erythrocytes by haemolytic Sendai virus particles. Fusion of Sendai virus particles with the plasma membrane of cells makes the membrane permeable to low molecular weight compounds and ions, and this leads to cell swelling [28,29]. At least for fusion that involves a virion acting as a bridge between two cells, the swelling is responsible for the rounding of already fused cells [30]. Thus, it was shown that the formation of giant cells was prevented when osmotic swelling was inhibited by hypertonic media [31,32]. Furthermore, when early-harvested (non-haemolytic and non-leaky) Sendai virions were used, giant cells were not formed [33,34]. That cell fusion nevertheless occurred under these circumstances was demonstrated by showing that the treated erythrocytes enlarged into giant cells on subsequent exposure to a hypotonic medium [35]. It was therefore concluded that osmotic swelling appears to be the driving force that results in cells, in which membrane-fusion sites are present, being able to expand into poly-erythrocytes. Work on the fusion by Sendai virus of human erythrocyte ghosts, which were prepared with or without sequestered macromolecules, such as bovine serum albumin or dextran, has also shown that colloidal osmotic swelling is responsible for the rounding-up of virally-fused ghosts [36]. Our findings on the role of osmotic swelling in the formation of giant cells from human erythrocytes that are exposed to electrical breakdown pulses are, therefore, fully consistent not only with the swelling properties of erythrocytes which have been permeabilised by electrical breakdown, but also with the formation of giant erythrocytes and poly-ghosts resulting from the action of the Sendai virus on intact and lysed erythrocytes, respectively.

By comparison with their responses to electrical breakdown in the presence of Mn^{2+}, Ca^{2+}, Ba^{2+}, Mg^{2+}, Na^+, K^+ and Al^{3+}, human erythrocytes behaved anomalously in the presence of Zn^{2+} with regard to lysis, fusion and cell swelling in our experiments. In relation to the ability of Zn^{2+} to inhibit the rapid cell shrinkage that otherwise ensued when erythrocytes were subjected to electrical breakdown in media of low-ionic strength, it may be relevant that Zn^{2+} is well-known to inhibit the leakage of cations, negatively-charged, and neutral molecules from cells that have suffered plasma membrane damage by haemolytic viruses, toxins, complement, and other cytotoxic agents [37]. Zn^{2+} is some 10-fold more effective than Ca^{2+} in this respect. It is also interesting that recent work on Na^+ channels in plasma membranes from mammalian heart ventricular muscle, which are blocked by Zn^{2+} with a approx. 100-fold higher affinity than other Na^+-channel subtypes, has indicated that Zn^{2+} appears to bind to a site that contains one or more cysteine sulphydryl groups [38]. Conceivably then, Zn^{2+} may inhibit the immediate loss of ions from erythrocytes that are subjected to electrical breakdown in low-ionic-strength media by interacting with cysteine groups in membrane proteins.

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