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Relationships between the surface exposure of acidic phospholipids and cell fusion in erythrocytes subjected to electrical breakdown

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The procoagulant activity of human erythrocytes, which provides a measure of the translocation of acidic phospholipids from the inner to the outer monolayer of the plasma membrane, has been compared with the percentage cell fusion in experiments on the effects of electrical breakdown pulses under differing experimental conditions. After treatment with breakdown pulses of 20 μs or longer (5 kV cm^{-1}), the plasma membranes of erythrocytes in 250 mM sucrose exhibited an almost complete loss of asymmetry with respect to acidic phospholipids. As the breakdown voltage was increased from 2 to 5 kV cm^{-1} (with breakdown pulses of 99 μs), the surface exposure of acidic phospholipids and cell fusion increased approximately in parallel. Furthermore, with 99 μs pulses and a voltage of 3 kV cm^{-1}, a decrease in the osmolarity from 250 to 150 mM of the sucrose medium was accompanied by an increase in both the surface exposure of acidic phospholipids and the extent of cell fusion. Breakdown pulses of 2–5 μs were sufficient to cause a marked loss of asymmetry, but no cell fusion was observed unless the pulse length was at least 20 μs. Kinetic experiments indicated that exposure of the acidic phospholipids at the cell surface was more likely to be due to a direct effect of the electric field pulses on plasma membrane structure than to secondary effects, such as the action of endogenous proteinases on the membrane skeleton. It seems possible that a localised, surface exposure of acidic phospholipids may contribute to the 'long-lived fusogenic state' (Sowers, A.E. (1986) J. Cell Biol. 102, 1358–1362) and the 'transient permeant structures' (Teissié, J. and Rols, M.P. (1986) Biochem. Biophys. Res. Commun. 140, 253–266) that enable cell fusion to occur when contact between cells is established after they have been subjected to field pulses. Our observations also provide circumstantial support for the concept that changes in the phospholipid asymmetry of membranes may be important in physiologically-occurring instances of biomembrane fusion.

Introduction

The phospholipids in the outer leaflet of the human erythrocyte plasma membrane are primarily phosphatidylcholine and sphingomyelin, while the inner leaflet contains the acidic phospholipids [1]. Exposure of acidic phospholipids on the outside of sickled erythrocytes [2], and platelets [3,4] yields procoagulant surfaces that facilitate the conversion of prothrombin into thrombin. The catalytic potential of such surfaces provides a semi-quantitative measure of the exposed acidic phospholipids [3–5]. In previous work, we observed that an increase in the procoagulant activity of human erythrocytes is associated with fusion of these cells that is induced by the permeant molecule poly(ethylene glycol) 400 in the presence of Ca^{2+}. Erythrocytes that were incubated with ionophore A23187, subtilisin, and Ca^{2+} also developed procoagulant activity, equivalent to a complete loss of phospholipid asymmetry, and they fused on subsequent exposure to a hypotonic medium. From these experiments we concluded that a translocation of phosphatidylserine to the outer leaflet of the plasma membrane plays an important role in fusion protocols that involve cell swelling [6].

Recent reports from other laboratories have also indicated that changes in the phospholipid asymmetry

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of plasma membranes are associated with cell fusion [7,8]. By contrast, although fusion of vesicular stomatitis virus with lipid-symmetric human erythrocyte ghosts is more rapid than fusion with lipid-asymmetric ghosts, the susceptibility to fusion appears to be related to the packing characteristics of the target membrane rather than to any particular phospholipid [9].

Some years ago Dressler et al. [10] found that the asymmetric distribution of phosphatidylethanolamine in the plasma membrane of human erythrocytes was completely lost in ghosts prepared by colloid-osmotic lysis after electric breakdown and resealing, although phosphatidylserine was much less affected. No experiments on the fusion of cells exposed to the electric breakdown pulses were reported, but a possible mechanistic link between the enhancement of phospholipid flip-flop and electrically-induced cell fusion was discussed. More recently it has been observed that electric field pulses induce the formation of a 'long-lived fusogenic state' [11] or 'transient permeant structures' [12] that enable cell fusion to occur when contact between cells is established after they have been subjected to field pulses. Changes in the phospholipid asymmetry of plasma membranes might contribute to such states or structures [13]. We have therefore undertaken experiments to investigate possible relationships between the surface exposure of acidic phospholipids and cell fusion in human erythrocytes that are exposed to electric field pulses.

Materials and Methods

Materials

Bovine blood coagulation factors (factor Xa, prothrombin, thrombin) were from Sigma Chemical Co. Bovine factor Va was from Diagnostic Reagents Ltd. The chromogenic substrate for thrombin, S2238, (H-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide dihydrochloride), was from KabiVitrum (Stockholm, Sweden).

Methods

Unless otherwise stated, assays for prothrombinase were performed in duplicate, and each experiment was undertaken at least three times. In each case, the results shown are from one representative experiment.

Exposure of erythrocytes to electrical breakdown pulses

Human erythrocytes were washed and freed from leucocytes as described in [14], 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 (150 or 250 mM), containing 1.1 mM Ca^{2+} and 10 mM histidine, which had a conductivity of 235 $\mu S cm^{-1}$. Ca^{2+} was present in the medium to stabilise the erythrocytes against the alternating field and the d.c. field pulses [15]. In the absence of Ca^{2+}, the cells lysed when the d.c. pulses were applied. A stainless steel pipetting annular electrode chamber (Kruss GmbH, Hamburg) with an electrode gap of 500 μm was used with a Zimmermann Cell Fusion System (GCA Corp., Chicago). 1.5 min after adding the packed cells to the buffered sucrose, the erythrocytes were aligned by dielectrophoresis in an a.c. field of 0.16 $kV cm^{-1}$ at 1.5 MHz. After a further 0.5 min, three square wave, electric field pulses (of 99 μs, unless otherwise described in the text) were applied to the cells at 1 s intervals, and the a.c. field was removed 0.5 min later. The pulsed cell sample was removed from the electrode chamber, and divided into two portions. One portion was used to investigate cell fusion qualitatively, the other portion was assayed for prothrombinase activity and cell lysis. To avoid possible interference by carboxyfluorescein in the prothrombinase assay, quantitative assays of cell fusion were made separately.

Assay of procoagulant activity and cell lysis

Samples (20 μl) of pulsed or unpulsed erythrocytes, or lysed cells, were added to 480 μl of the above buffered sucrose solution. 100 μl samples were then removed for the determination of procoagulant activity. 50 μl of a cocktail (containing factor Xa, factor Va, and $CaCl_2$) was added to each sample, and the mixture was incubated at 37°C for 2 min before adding 50 μl of a pre-warmed solution containing prothrombin and $CaCl_2$. The final concentrations in the assay were: 2 μM prothrombin, 4 mM $CaCl_2$, 0.2 units per ml factor Xa, and 6 nM factor Va. Unless otherwise stated in the text, prothrombin was added to the cells 6 min after the electrical breakdown pulses. After various subsequent time intervals, 20 μl samples were removed, and thrombin formation was stopped by diluting them into 0.5 ml 50 mM Tris-HCl, 120 mM NaCl, 2 mM EDTA (pH 7.5) at room temperature. Chromogenic substrate S2238 was added to a final concentration of 150 μM, and the quantity of thrombin present was determined from the rate of change of absorbance at 405 nm using a calibration curve.

The remains of each sample of pulsed or unpulsed cell sample was maintained at 37°C for 11 min (until the prothrombinase assays were completed), 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 totalled lysed, by adding 5 μl of packed cells to 950 μl of distilled water, and subsequently sonicated. The preparations of lysed cells that were obtained in this way gave values in the assay

for the maximum procoagulant activity which were reproducible throughout the course of each experiment.

Cell fusion

Erythrocytes were labelled with 6-carboxyfluorescein as previously described [16], 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 [17]. 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 epifluorescence. Cells were counted on a microscope slide in randomly selected fields after their exposure to breakdown pulses. Between 150 and 450 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.

Results

Haemolysis, procoagulant activity, and fusion of erythrocytes exposed to breakdown pulses of increasing duration

Intact human erythrocytes in sucrose solutions had virtually no procoagulant activity (3.1% ± S.D. 2.1, n = 9, of the procoagulant activity of lysed cells). When erythrocytes were incubated for 45 min at 37°C in sucrose solutions of decreasing osmolarity in previous work, their procoagulant activities increased in parallel with the cell lysis as a consequence of the increasing accessibility of the acidic phospholipids in the inner leaflet of the plasma membrane [6]. Erythrocytes are permeabilised by electrical breakdown pulses, and this results in colloid osmotic swelling that is followed by cell lysis [18]. Consequently, erythrocytes that have been exposed to electrical breakdown pulses similarly exhibit procoagulant activity. In order to see if acidic phospholipids become accessible in pulsed cells as a primary consequence of electrical breakdown, rather than as a secondary consequence of cell lysis, it is desirable to minimise colloid osmotic swelling. A minimal release of haemoglobin (M_r 65 000) also indicates

TABLE I

Colloid osmotic haemolysis of human erythrocytes after exposure to electrical breakdown pulses in differing non-ionic media

The incidence of haemolysis, ± S.D. (n = 3), was determined 23 min after human erythrocytes were aligned by dielectrophoresis and exposed to three consecutive d.c. square wave pulses (5 kV cm^{-1}) in 250 mM solutions of the solutes shown, as described in Materials and Methods. Each solution contained 1.1 mM Ca^{2+} and 10 mM histidine, and had a conductivity of 235 μS cm^{-1}.

Solution	Duration of pulses (μs)	Haemolysis (%)
250 mM erythritol	20	65.7 ± 4.5
250 mM mannitol	20	27.6 ± 4.0
250 mM sucrose	20	10.1 ± 1.1
250 mM erythritol	99	55.8 ± 2.5
250 mM mannitol	99	31.6 ± 4.3
250 mM sucrose	99	17.2 ± 2.1

that factor Va (M_r 330 000), used in the prothrombinase assay, is unlikely to have entered the pulsed cells. Minimal swelling and haemolysis were achieved by an appropriate choice of non-electrolyte in the medium used for pulsing the cells. Table I shows the percentage haemolysis observed, under the conditions of our experiments, 23 min after erythrocytes were subjected to three consecutive d.c. square wave pulses (5 kV cm^{-1}) of 20 μs or 99 μs duration, in 250 mM solutions (conductivity 235 μS cm^{-1}) of erythritol, mannitol, or sucrose (each containing 10 mM histidine and 1.1 mM Ca^{2+}). Although solutions of mannitol and erythritol are commonly used in the electrofusion of cells, it is clear that haemolysis of the treated erythrocytes was least in the sucrose solution. Erythrocytes were therefore pulsed in sucrose solutions in all of the experiments reported in the present paper.

The effect of electrical breakdown pulses (5 kV cm^{-1}) of increasing duration, from 0.5 to 99 μs, on the procoagulant activity and the haemolysis of erythrocytes suspended in 250 mM sucrose solution was investigated. Fig. 1 shows that the percentage procoagulant activity of the cells increased much more rapidly than the percentage lysis for pulse lengths between 0.5 μs and 20 μs. Furthermore, with 60 μs pulses about 58% of the total prothrombinase activity was exposed although, judging from the percentage haemolysis, only about 15% of the total prothrombinase activity was attributable to acidic phospholipids in the inner monolayer of lysed cells. Approximately 43% of the acidic phospholipids were therefore exposed at the surface of the treated cells. However, for reasons considered below in the Discussion section, this is probably an underestimate of the loss of phospholipid asymmetry in the pulsed cells.

With a breakdown voltage of 5 kV cm^{-1}, pulses of only 2 μs duration were sufficient to induce a marked

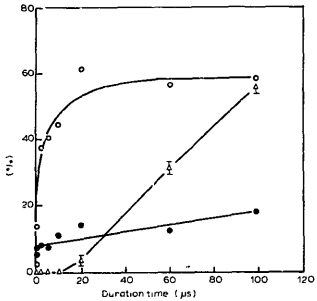

Fig. 1. Human erythrocytes in a 250 mM sucrose solution, containing 1.1 mM Ca^{2+} and 10 mM histidine, were aligned in pearl chains by dielectrophoresis and subjected to three consecutive d.c. square wave pulses (5 $kV cm^{-1}$), of increasing duration time (0.5 to 99 μs), as described in Materials and Methods. The percentage procoagulant activity (○), percentage cell fusion (△), and percentage haemolysis (●) were determined as described in Materials and Methods.

loss of phospholipid asymmetry in the treated erythrocytes (Fig. 1). Interestingly, much longer pulses were required for the induction of cell fusion (Fig. 1). It is thus apparent that in addition to the surface exposure of acidic phospholipids, other structural changes in the erythrocyte membrane, which are induced by relatively long electrical pulses, precede cell fusion.

Effects of the breakdown voltage on procoagulant activity, lysis, and cell fusion

Using 99 μs pulses, experiments were undertaken to study the effect of varying the d.c. breakdown voltage, over the range 2–5 $kV cm^{-1}$, on the procoagulant activity, lysis, and fusion of erythrocytes in 250 mM sucrose. As with increases in the duration of the field pulses, increases in the breakdown voltage resulted in the percentage procoagulant activity rising more rapidly than the percentage haemolysis (Fig. 2). With an applied voltage of 3 $kV cm^{-1}$, 45% of the total prothrombinase activity was exposed. However, as judged by the percentage haemolysis, only about 18% of the total prothrombinase activity was attributable to phospholipids in the inner monolayer of lysed cells. This further indicates that electrical breakdown pulses act on erythrocytes to expose acidic phospholipids at the cell surface.

With a breakdown voltage of 2 $kV cm^{-1}$, no cell fusion occurred. As the voltage was increased, chains of fused cells of increasing length were seen. Unlike the dissimilar effects of increases in pulse length on the percentage procoagulant activity and the percentage cell fusion (Fig. 1), it is apparent from Fig. 2 that the percentage cell fusion and the procoagulant activity increased in a comparable manner when the breakdown voltage was increased from 2.4 to 5 $kV cm^{-1}$.

Effect of hypotonic sucrose solution

Previous work in this laboratory showed that chains of up to 14 fused cells were formed when human erythrocytes in 150 mM erythritol were exposed to an electric field pulse, whereas only a few cells were fused when they were pulsed in 200 mM erythritol [17]; NS1 mouse myeloma cells behaved in a similar manner [19]. In electro-acoustic fusion of human erythrocytes, the fusion yield in 170 mosM solutions was much higher than the yield from cells in 272 mosM solutions [20]. The electrofusion of mammalian cells in strongly hypo-osmolar media containing sorbitol also resulted in high yields of hybridoma cells [21]. We have therefore investigated the effect of varying the d.c. breakdown voltage, over the range 2 to 5 $kV cm^{-1}$, on the procoagulant activity, lysis, and fusion of erythrocytes in 150 mM sucrose solutions.

Although the effect of increasing the applied voltage on the prothrombinase activity of erythrocytes in 150 mM sucrose was broadly similar to that seen with the cells in 250 mM sucrose (Fig. 2), there was some indication that the prothrombinase activity of the cells might develop at slightly lower voltages in the hypotonic solutions (Fig. 3). This possibility was investigated

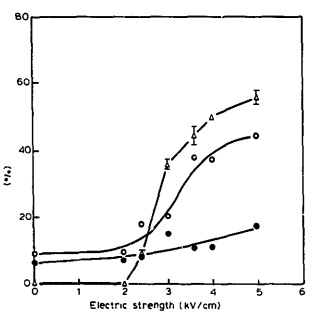

Fig. 2. Human erythrocytes in a 250 mM sucrose solution, containing 1.1 mM Ca^{2+} and 10 mM histidine, were aligned in pearl chains by dielectrophoresis and subjected to three consecutive d.c. square wave pulses (99 μs), of increasing voltage (2 to 5 $kV cm^{-1}$), as described in Materials and Methods. The percentage procoagulant activity (○), percentage cell fusion (△), and percentage haemolysis (●) were determined as described in Materials and Methods.

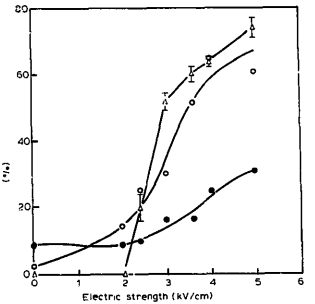

Fig. 3. Human erythrocytes in a 150 mM sucrose solution, containing 1.1 mM Ca^{2+} and 10 mM histidine, were aligned in pearl chains by dielectrophoresis and subjected to three consecutive d.c. square wave pulses (99 μs), of increasing voltage (2 to 5 kV cm^{-1}), as described in Materials and Methods. The percentage procoagulant activity (○), percentage cell fusion (△), and percentage haemolysis (●) were determined as described in Materials and Methods.

by measuring the prothrombinase activity and the haemolysis in triplicate with erythrocytes from a single donor that were subjected to pulses of 99 μs (3 kV cm^{-1}) in 150 mM and 250 mM sucrose solutions. It appears from Table II that, after allowing for the lysed cells, more procoagulant activity was exposed in the erythrocytes that were subjected to the breakdown pulses in the hypotonic sucrose solution.

Longer chains of fused cells were observed with erythrocytes in the hypotonic sucrose solution than with cells in 250 mM sucrose. With erythrocytes in 150 mM sucrose solution (Fig. 3), as with the cells in 250 mM sucrose (Fig. 2), the percentage cell fusion and the percentage procoagulant activity increased in an approximately parallel manner when the breakdown voltage was increased from 2.4 to 5 kV cm^{-1}.

TABLE II

Prothrombinase activity of human erythrocytes after exposure to electrical breakdown pulses in sucrose solutions of differing osmolarity

The percentage prothrombinase activity and the percentage haemolysis were determined in triplicate for erythrocytes from a single donor that were aligned by dielectrophoresis and exposed to three consecutive d.c. 99 μs square wave pulses (3 kV cm^{-1}) in 150 mM and 250 mM solutions of sucrose (containing 1.1 mM Ca^{2+} and 10 mM histidine), as described in Materials and Methods.

Solution	% prothrombinase activity	% haemolysis
150 mM sucrose	25.7	11.4
	21.3	11.7
	24.3	12.2
	(mean 23.8 ± S.D. 1.8)	(mean 11.8 ± S.D. 0.3)
250 mM sucrose	15.2	12.2
	14.0	7.7
	10.2	8.8
	(mean 13.1 ± S.D. 2.1)	(mean 9.6 ± S.D. 1.9)

TABLE III

Prothrombinase activity of human erythrocytes at differing times after exposure to electrical breakdown pulses

The percentage prothrombinase activity and the percentage haemolysis were determined, in triplicate, for aligned erythrocytes from a single donor 6 min and 36 min after their exposure to three consecutive d.c. 99 μs square wave pulses (4 kV cm^{-1}) in 150 mM sucrose (containing 1.1 mM Ca^{2+} and 10 mM histidine), as described in Materials and Methods.

Time period	Prothrombinase (nmol thrombin l^{-1} min^{-1})	% prothrombinase	% lysis
6 min	10.9	41.5	19.1
36 min	11.6	44.5	31.5
6 min	13.7	52.4	18.6
36 min	14.0	53.4	26.6
6 min	14.7	56.1	15.1
36 min	16.4	62.7	26.4
36 min (untreated cells)	1.4	5.3	6.6
Lysed cells	26.1		

Time course of exposure of acidic phospholipids

If degradation of the membrane skeleton by endogenous proteases is responsible for the development of procoagulant activity in plasma membranes exposed to electrical breakdown pulses, the procoagulant activity might be expected to increase with time after delivery of the pulses. The procoagulant activity was therefore determined (in triplicate) 6 min and 36 min after the breakdown pulse with erythrocytes from a single donor that were subjected to 99 μs pulses (4 kV cm^{-1}) in 150 mM sucrose solution and then maintained at room temperature. Table III shows the data obtained from which it is apparent that there was little increase in surface exposure of acidic phospholipids between 6 min and 36 min after the field pulses; the mean value of the procoagulant activity after 36 min was only 8% greater than that after 6 min. This indicates that exposure of the acidic phospholipids at the cell surface was more likely to be due to a direct effect of the electric field pulses on plasma membrane structure than to secondary effects, such as the action of endogenous proteinases on the membrane skeleton.

The percentage haemolysis in Table III increased with time; the mean value of the percentage haemolysis after 36 min was 60% greater than that after 6 min.

Since the haemolysis, but not the procoagulant activity of the cells increased markedly between 6 and 36 min after the field pulses, it would seem that the cells which lysed during this period also resealed almost completely.

Discussion

The rate of conversion of prothrombin to thrombin by the enzyme complex factor Xa-factor Va, which we employed in our experiments, has been used quite extensively as a convenient, sensitive, and semi-quantitative way of monitoring the surface exposure of phosphatidylserine in the plasma membranes of platelets [22] and erythrocytes [2]. In the latter work, it was shown that a combination of sickling and ATP depletion resulted in a time-dependent increase in phosphatidylserine in the outer membrane leaflet of human erythrocytes, as measured by phospholipase A_2 accessibility and by the prothrombinase assay. However, it was recently reported that the prothrombinase assay detects phosphatidylethanolamine almost as well as phosphatidylserine in mixtures with phosphatidylcholine [5]. The relative reactivity in the prothrombinase assay of the acidic phospholipids when they are in the erythrocyte plasma membrane is nevertheless unknown. There is therefore no conflict between our observations and the earlier findings of Dressler et al. [10].

Pronase was included in the fusion medium in previous work from this laboratory on the electrofusion of erythrocytes [23], since an early investigation showed that pronase stabilises cells against lysis caused by electrical breakdown [24]. This appears to result from proteolytic action on the cells and the presence of contaminating Ca^{2+} ions in commercial preparations of pronase [15]. Since Ca^{2+} ions alone substantially protect human erythrocytes against electrical breakdown, and it was desirable to avoid the proteolytic effects of pronase in the present experiments, a pronase-free medium that contained 1.1 mM Ca^{2+} was used in the work reported here. Cell fusion occurred similarly in the presence of Mg^{2+} (1.1 mM). However, Mg^{2+} was less effective than Ca^{2+} in protecting against cell lysis, and this precluded experiments on possible changes in phospholipid asymmetry being investigated in the presence of Mg^{2+}.

Earlier analyses of changes in phospholipid asymmetry associated with electric breakdown were made on ghosts after they had been incubated for 60 min [10]. It is therefore possible that the observed changes may have arisen from secondary effects, such as the action of endogenous proteinases, rather than from a direct effect of the electric field pulses on membrane structure. Our analyses were commenced only 6 min after exposure of the cells to breakdown pulses, and there was only a small increase in procoagulant activity during the following 30 min. Furthermore, our measurements were on cell suspensions in which the percentage haemolysis was mostly less than 20% (with the 10 μs pulses in Fig. 1, haemolysis was only 10%). Secondary effects cannot be completely excluded, however, as we were unable to determine whether changes in phospholipid asymmetry occurred during the first 6 min. after the pulses.

In our experiments partial resealing of lysed erythrocytes prior to commencing our prothrombinase assays, may have resulted in acidic phospholipids in the inner monolayer regaining their inaccessibility. The contribution to the procoagulant activity that was attributed to acidic phospholipids in the inner monolayer, as judged from the percentage haemolysis, could therefore have been overestimated (particularly as the extent of haemolysis was determined after the prothrombinase assays were completed). Consequently, in the experiment of Fig. 1, there may have been an almost complete loss of asymmetry in the distribution of acidic phospholipids in erythrocytes subjected to breakdown pulses longer than about 20 μs.

In the plasma membranes of eukaryotes, aminophospholipid asymmetry appears to be due to the ATP-dependent aminophospholipid translocase activity that counterbalances the spontaneous lipid randomization by flip-flop [25]. Interactions of the acidic phospholipids with the membrane skeleton may also contribute to the maintenance of phospholipid asymmetry in erythrocytes [25], in which case damage to the membrane skeleton caused by field pulses might be expected to give rise to a loss of phospholipid asymmetry. Voltage-dependent blebbing of the plasma membrane that was inhibited by increasing the tonicity of the medium has been observed by light microscopy with several cell types exposed to electric field pulses, and it has been suggested that this phenomenon may have resulted from a local rupture of the cytoskeleton [26]. Although no blebbing was observed with human erythrocytes subjected to electric field pulses, it may be relevant that the loss of phospholipid asymmetry in human erythrocytes which occurs when they are treated with Ca^{2+} and ionophore A23187 is apparently associated with shedding of vesicles from the plasma membrane [27].

It is relevant to our findings that the application of electrical pulses to yeast cells has been observed to induce an increase in absorption of the cationic dye, 9-aminoacridine. This may have been due to the appearance of additional electronegative groups on the outer side of the plasma membrane [28]. A study made with ^{31}P-NMR of electropermeabilised Chinese hamster ovary cells also showed that the polar head groups of about 70% of plasma membrane phospholipids were in a new configuration in the permeabilised cells, and

it has been proposed that this leads to a decrease in hydration repulsion forces between the treated cells that facilitates cell fusion [29,30].

In our experiments, breakdown pulses of 2–5 μs caused a marked loss of phospholipid asymmetry, but no cell fusion was observed unless the pulse length was at least 20 μs (Fig. 1). A loss of phospholipid asymmetry in the plasma membrane is therefore not sufficient to induce fusion. However, human erythrocytes that have apparently lost their phospholipid asymmetry have been found to exhibit an increased susceptibility to fusion induced by poly(ethylene glycol) 6000 [7]. Surface exposure of acidic phospholipids also occurs in the fusion of human erythrocytes induced by protocols that involves cell swelling [6]. Since we observed approximately parallel increases in the surface exposure of acidic phospholipids and cell fusion with breakdown pulses of 99 μs, it seems probable that exposure of acidic phospholipids at the cell surface facilitates the fusion process but that fusion cannot occur in the absence of other events, e.g. the development of structural defects in the phospholipid bilayer.

The primary work involved in bringing phospholipid bilayers into close apposition is that necessary to dehydrate their polar head groups. Hydration repulsion between bilayers of anionic phospholipids is readily overcome, however, by the addition of Ca^{2+} ions to the aqueous phase. Most of the water between the two membrane surfaces is then displaced, since the Ca^{2+} ions bind to the polar groups of the anionic phospholipids, and the membranes collapse together [31]. Movement of phosphatidylserine from the inner to the outer monolayer of the plasma membrane of erythrocytes in the presence of Ca^{2+} ions would thus be expected to decrease the forces of hydration repulsion between approaching cells, and it has been suggested that the fusogenic state of the plasma membrane that results from the exposure of cells to electrical field pulses may involve a loss of phospholipid asymmetry [13]. Our observations are consistent with this suggestion. A localised, surface exposure of acidic phospholipids may therefore contribute to the 'long-lived fusogenic state' [11] and the 'transient permeant structures' [12] that enable cell fusion to occur when contact between cells is established after they have been subjected to field pulses. However, the long-lived fusogenic state has a much shorter life-time [32] than the change in phospholipid asymmetry observed in our experiments. This would be anticipated if the surface exposure of acidic phospholipids is only one of several factors that contribute to the fusogenic state.

The transient pores induced by electroporation in the membranes of erythrocytes [33], the transient point defects which precede the electro-fusion of erythrocytes [34], and the similar but more stable defects which are associated with the electrically-induced, hemi-fusion of these cells [23], may correspond physically to sites at which phospholipid asymmetry has been lost. A surface exposure of phosphatidylserine at localised sites in the plasma membranes of erythrocytes as a consequence of electrical breakdown would additionally be expected to facilitate cell fusion in view of the fact that the addition of sufficient phosphatidylserine to liposomes composed of phosphatidylcholine leads to membrane fusion in the presence of Ca^{2+} ions [35]. The present findings thus give circumstantial support to the concept that changes in phospholipid asymmetry may be important in physiologically-occurring instances of biomembrane fusion, such as the fusion of myoblasts [36] and the exocytosis of chromaffin granules [37].

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