Biochimica et Biophysica Acta, 1025 (1990) 247–251
Elsevier

BBAMEM 70657

BBA Report

Low concentrations of macromolecular solutes significantly affect electrofusion yield in erythrocyte ghosts

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(Received 3 October 1989)
(Revised manuscript received 8 February 1990)

Key words: Membrane fusion; Electrofusion; Electroporation; Erythrocyte ghost; (Rabbit)

Electrofusion yields in rabbit erythrocyte ghosts containing various amounts of hemoglobin, bovine serum albumin, or dextran at low concentrations were measured as a function of pulse field strength and pulse decay half-time. The presence of any of the macromolecules in low concentrations caused fusion yields to be significantly higher than when the ghosts were white (i.e., containing only buffer). The fusion yield enhancement was also critically dependent on the parameters of the electric field pulse. The fusion yield was also significantly affected by small changes in the concentration of hemoglobin when it was present outside the ghost membranes in the suspension buffer.

The use of an electric field pulse as a fusogen (electrofusion) [3] has three unique characteristics. It allows membrane fusion to be induced: (i) at will, (ii) non-chemically, and (iii) with a high enough probability that sigle events can be observed in real time. The use of dielectrophoresis [4] is a unique method of inducing close membrane-membrane contact because it is a mild, non-chemical, and completely reversible phenomenon. Use of dielectrophoresis and electrofusion offers the possibility of membrane fusion studies in which the chemistry of the medium, the induction of close membrane-membrane contact, and the induction of fusion can be manipulated independently of one another.

Although the electrofusion mechanism is poorly understood at present, the discovery of factors which have strong modulating effects on fusion yield may be expected to provide important clues which may lead to a better understanding of the mechanism in the future. I report in this paper that low concentrations of macromolecular solutes have a major effect on electrofusion yield in rabbit erythrocyte ghosts.

White and dark red rabbit erythrocyte ghosts [5] were prepared as follows. Approximately 10 ml of New Zealand White rabbit whole blood was collected by venipuncture into a test tube containing 0.3 ml of 1 M sodium citrate. Isotonic phosphate buffer (32 ml, pH 7.4) was added to a 2.5 ml aliquot of this blood and centrifuged at 1200 rpm ($150 \times g$) for 10 min. After removing the buffy coat and supernatant, the pellet was resuspended with 32 ml of hemolysis buffer (5 mM sodium phosphate (NaP_i) buffer, pH 8.5), to cause hemolysis, and then allowed to stand for 20 min, to allow further dilution of the intracellular hemoglobin concentration. This suspension was then centrifuged ($10\,000 \times g$ for 20 min). The supernatant was removed and the pellet resuspended with wash buffer (20 mM NaP_i buffer, pH 8.5) and centrifuged again and then labeled with 1,1′-dihexadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) as previously described [6]. This preparation of erythrocyte ghosts yielded a dark red pellet when centrifuged. Pellets of white erythrocyte ghosts were obtained by following the same procedure except that the pellet, obtained after the hemolysis step, was resuspended with more hemolysis buffer (5 mM NaP_i) instead of wash buffer and allowed to stand for 20 min and then pelleted and then washed with the 20 mM buffer. All operations were conducted at 0–4°C. The rabbits were fed the NIH-09 open formula rabbit diet (purchase specification NIH-11-136f: Dr. J. Knapka, NIH, Bethesda, personal communications) and commercially supplied by Zeigler Bros., Inc. (Gardners, PA 17324-0095). The 24 h unit gravity hematocrit of the rabbit blood was about 0.6.

The preparation of erythrocyte ghosts with other hemoglobin concentrations was carried out as follows. The above procedure was modified as follows. Hemolysis buffer (35 ml) was added to 1.5 ml of a rabbit

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erythrocyte pellet (obtained after one wash of the erythrocytes in isotonic buffer). For light pink ghosts, distilled water (30 ml) was added to a 1.5 ml pellet of washed erythrocytes and immediately centrifuged. The supernatant was removed and the pellet resuspended with 6 ml of 10 mM NaP_i (pH 8.5) and centrifuged. Then the supernatant was removed and replaced with a 6 ml of 10 mM NaP_i and incubated for 10 min and then 0.75 ml of 0.2 M sodium phosphate was added and incubated for 1 h at 25 °C and then followed by washes in wash buffer. For dark pink ghosts, the hemolysis step involved resuspending erythrocytes with hemolysis buffer and incubation for 1 h before washes in the 20 mM buffer. For light red ghosts, the hemolysis step involved resuspended the erythrocytes with hemolysis buffer for 30 min before the washes in the 20 mM buffer.

The hemoglobin concentration in the ghosts membranes was measured as follows. Pellets of intact erythrocytes were resuspended with isotonic phosphate to obtain an erythrocytes suspension with a cell concentration which was 1/10 of that of the pellet. An aliquot (0.45 ml) of this diluted pellet was vortexed with 0.05 ml of a 20% Triton X-100 solution. The resulting solution was diluted further by a factor of 100 and the optical density, measured colorimetrically at 420 nm, was taken as the 100% hemoglobin concentration. Pure buffer was used to set the OD = 0.0 (= 100% T) point. Residual hemoglobin in washed erythrocyte ghosts was measured in an identical fashion except that the ghosts were in 20 mM NaP_i buffer and the OD measurements used to calculate hemoglobin concentration as a percent of that in intact erythrocytes.

Hemoglobin was reloaded back into rabbit white erythrocyte ghosts as follows. Rabbit erythrocyte hemolysate (supernatant from first spin after hemolysis) was extensively dialyzed against 2 mM NaP_i (pH 8.5). Then, a 1.0 ml pellet of white ghosts was incubated with 9.0 ml of the dialyzed hemolysate for 30 min at 0–4 °C. Then, 10 ml of 40 mM NaP_i was added and incubated at 5 °C for 1 h, and was followed by two washes in 20 mM NaP_i. The final spin yielded a pellet with an appropriate shade of color and a clear supernatant.

Bovine serum albumin (Sigma, fraction V) or Dextran T-70 (Pharmacia) was reloaded back into white erythrocyte ghosts as follows. NaP_i buffer (2 mM, pH 8.5) containing bovine serum albumin, or the dextran, at concentrations of 0.1%, 0.5%, and 1.0% (m/v). To 0.6 ml of white ghost pellet was added 5.4 ml of 0.5% or 1.0% of the solute-containing buffer and incubated on ice for 30 min. Then 6 ml of 40 mM NaP_i also containing 0.5% or 1.0% of the solute was added and then incubated for 1 h at 25 °C. This solution was washed two times with 20 mM NaP_i. To make solute-loaded ghosts at the 0.1% concentration required that 9.0 ml of a 0.1% solute in 2 mM buffer be added to a 1.0 ml pellet of white ghosts in 20 mM NaP_i and incubated for 1 h on ice. Then 10 ml of 40 mM NaPi also containing the solute (0.1%) were added and incubated for 1 h at 25 °C followed by two washes in 20 mM NaP_i buffer.

The effect of hemoglobin in the extracellular compartment was explored as follows. White and dark red rabbit erythrocyte ghosts were prepared as above and resuspended in 20 mM NaP_i containing the hemolysate (= 1 × hemoglobin concentration in Fig. 3) from the first hemolysis or 20 mM NaP_i buffer was added to the hemolysate, to vary the concentration of hemoglobin without changing buffer strength or pH, and then used to resuspend the ghosts.

The fusion protocol and fusion yield calculation were conducted as follows. For any experimental condition all ghost membranes were made from the same preparation. The only difference between the membranes was the fact that some (about 1 in 15) were DiI-labeled and the remainder were unlabeled. All ghost membranes were aligned into pearl chains with a low strength (10–15 V/mm) alternating current (60 Hz) and then subjected to a single direct current, exponentially decaying pulse from a device which was previously described (Fig. 3 in Ref. 2). In all cases except one, the ghost membranes fusion assay took place with membranes in 20 mM NaP_i buffer (pH 8.5). In one experiment the hemolysate (supernatant) from the preparation of pink ghosts (see above) was used intact, or diluted with additional 20 mM NaP_i buffer and used, to resuspend white or dark red ghost pellets to determine the effect of low concentrations of solute on the extracellular side of the membrane. The decay half-time of the pulses was monitored by a storage screen oscilloscope. In all cases, except for the DiI-labeling procedure, all membranes to be fused were identical. Fusion yield (FY) was calculated from two categories of counts made after the pulse was applied: (i) single (i.e., unfused) fluorescent ghost membranes (N_s), and (ii) two or more (i.e., fused) adjacent fluorescent membranes (N_m), and use of the formula $FY = [(N_m)/(N_s + N_m)] \times 100$.

Fig. 1 shows that over a broad range in pulse field strengths (E) and pulse decay half-times ($T_{1/2}$) the fusion yields in dark red ghosts are almost always significantly higher than when the ghosts are white. The exceptions are at a pulse field strength of 300 V/mm and the decay half-time is less than 1.0 ms and at a pulse strength of 500 V/mm and the decay half-time is 1.0 ms or longer. The level of residual hemoglobin in the dark red and white ghosts was not measured since both the visual impression of 'pinkness' and the pattern in fusion yeilds (compared to white ghosts) were reproducible from one preparation to the next.

Fig. 2 shows that a change in the residual hemoglobin concentration from 0.07% (white) to 0.26% (light pink) had a strong enhancing effect on fusion yield when the pulse field strength was between 350 V/mm

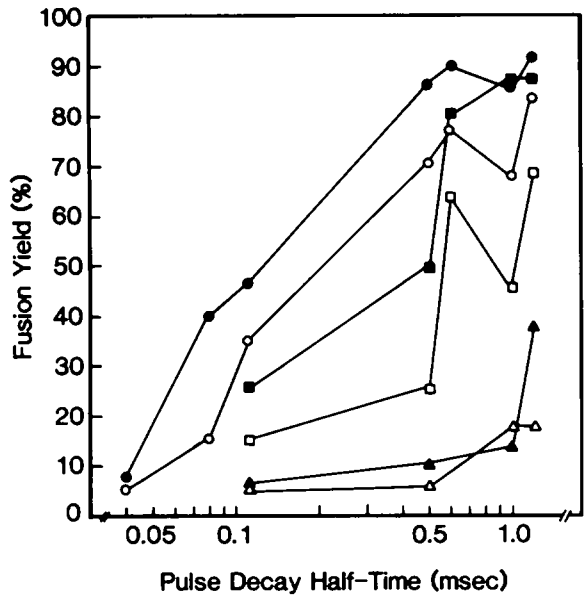

Fig. 1. Yields in white (△, □, ○) and dark red (▲, ■, ●) rabbit ghost + rabbit ghost fusions as a function of pulse decay half-time and pulse field strength (V/mm): △, ▲: 300; □, ■: 400; ○, ●: 500. The residual hemoglobin concentration in the ghosts was 4.8% of intact erythrocytes (see text).

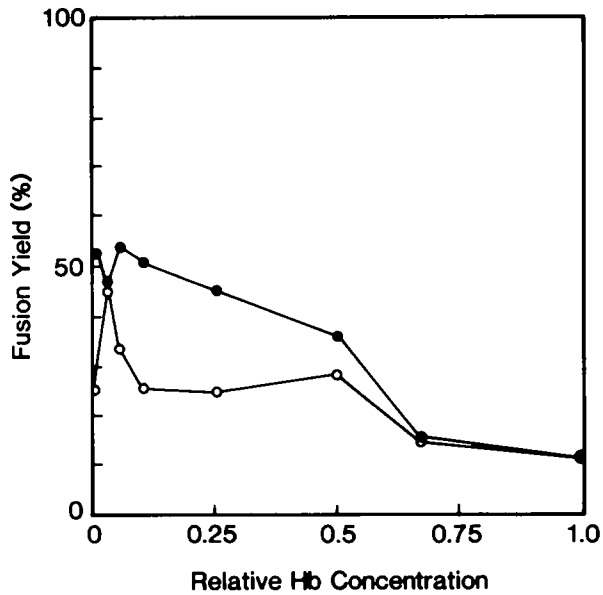

Fig. 3. Yields in dark red (●) and white (○) rabbit ghost + rabbit ghost fusions with a pulse field strength of 400 V/mm and a pulse decay half-time of 1.0 ms, as a function of hemoglobin concentration in the extracellular compartment (1.0 = hemolysate concentration from preparation of pin ghosts (see text)).

and 450 V/mm and the pulse decay half-time was 1.0 ms. For a 350 V/mm pulse the maximum increase is 37/10 or a factor of almost 4.

Fig. 3 shows that the yield in white ghost fusions and dark red ghost fusion is strongly influenced by the presence of hemoglobin in the extracytoplasmic compartment. White ghost and dark red ghost fusion yields were lowest when the extracellular hemoglobin concentration was the same as the hemolysate. Fusion in white ghosts is about two time higher over a broad range of low hemoglobin concentrations (0–0.5) and about three times higher over a narrow range of hemoglobin concentration of about 0.03–0.05 of hemolysate concentration). Conversely, yields in dark red ghost, compared to yields at a hemoglobin concentration of hemolysate (1.0 in Fig. 3), were enhanced by a factor of 2–3 over a broad range in hemoglobin concentration from 0 to about 0.5 of hemolysate concentration.

Table I shows that reloading white ghosts with hemoglobin causes a qualitative effect on fusion yield which is similar to that found when ghosts are prepared without a complete removal of the hemoglobin.

Table II shows that reloading white ghosts to a relatively low concentration with either bovine serum albumin or dextran also causes a general enhancement in fusion yield at the same pulse field strength (400 V/mm). The only specific exception to this enhancement effect is for the 400 V/mm data when the fusion yield at 0.1% dextran (fusion yield = 29%) is compared with the 'white' ghost control (fusion yield = 43%) for

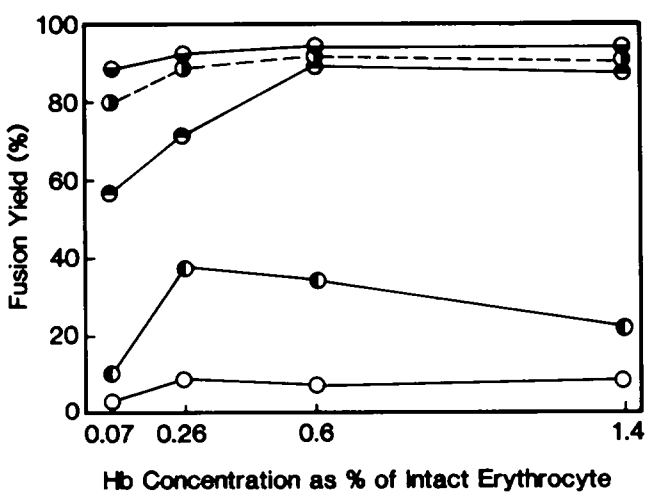

Fig. 2. Yields in rabbit ghost + rabbit ghost fusions with pulse decay half-time of 1.0 ms, and as a function of hemoglobin concentration (%) in the cytoplasmic compartment (intact erythrocyte = 100%). Pulse field strengths (V/mm): ○, 300; ◐, 350; ⊖, 400; ◑, 450; and ⊖, 500.

TABLE I

Effect on fusion yield of reloading white ghosts with hemoglobin

Pulse voltage [b]	Fusion yield (%), pellet color [a]	
	white	color
500	81	88
400	24	64
300	4	4

[a] Measured spectrophotometrically (see Methods): white, 0.07%; color, 0.6%.

[b] Decay half-time: 1.0 ms.

TABLE II

Effect on fusion yield of loading BSA or dextran T-70 into cytoplasmic compartment of white ghosts

Pulse voltage [c]	Fusion yields [a] (%)							
	BSA				Dextran			
	white	0.1%	0.5%	1.0%	White	0.1%	0.5%	1.0%
500	89	95	90	86	83	86	93	92
400	24	79	62	36	43	29	84	86
300	2	1	1	3	n.d.	2	14	3

[a] Experimental errors in fusion yield measurement are typically < 3–5% (see Fig. 5, Ref. 21).

[b] Cytoplasmic compartment concentration assumed to have equilibrated with concentration in extracellular compartment. The experimental error in preparing these concentrations is estimated to be < 1%.

[c] 1.0 ms decay half-time.

the dextran experiment; in almost all other comparisons with white ghosts, further increases in macromolecular concentrations are accompanied by further increases in fusion yield.

Our data show that fusion yield was higher in dark red ghosts than in white ghosts across a wide range of pulse field strengths and decay half-times (Fig. 1). This suggests that the presence of hemoglobin in the cytoplasmic compartments can play a significant modulating role in the membrane fusion process.

When ghosts were resealed after the release of specific amounts of hemoglobin, the hemoglobin-dependent enhancement of fusion yield was found to have its greatest effect at 0.26% hemoglobin for 350 V/mm pulses and at 0.6% hemoglobin for 400 V/mm pulses (Fig. 2). There is little effect on fusion yields at higher hemoglobin concentrations. At higher or lower pulse voltages the fusion yield is, respectively, saturated or too low to obtain practical measurements and little effect was observed (Fig. 2). This suggests that there is a specific relationship between the electric field pulse strength and the hemoglobin-enhancing fusion yield effect. The determination of the effect of varying $T_{1/2}$ was beyond the scope of this paper.

The fact that the fusion yields were similarly enhanced when hemoglobin was reloaded into white ghosts (Table I) suggests that the fusion yield enhancement is not a artifact of ghost preparation. Furthermore, the fact that fusion yield enhancements are observed when bovine serum albumin or T-70 dextran are used instead of hemoglobin (Table II) suggests that the effect is not due to a specific protein, but to a solute macromolecule. Lastly, the observations that yields in white ghost + white ghost fusions are strongly enhanced at a very narrow range in low extracellular hemoglobin concentrations while yields in dark red + dark red fusions are strongly enhanced over a broad range in hemoglobin concentrations (Fig. 3) also suggests that large solute molecules are perturbing the fusion process and that the solute macromolecule effect can operate from either or both sides of the ghost membranes. Assuming a hematocrit of 0.6, a mean corpuscular hemoglobin concentration of 0.33 [7], and a dilution of a 2.5 ml rabbit pellet into a volume of 2.5 + 34.5 ml of buffer, a 1.0 × hemolysate calculates to an extracellular hemoglobin concentration of 0.495 g hemoglobin per 34.5 ml of solution or, assuming a hemoglobin molecular mass of 66 kDa, a concentration of 0.4 mM. Since the bovine serum albumin and the dextran were close or relatively close to the hemoglobin molecular weight the large effect on fusion is due to a very small concentration of macromolecular solute compared to the concentration of NaP_i in the buffer (20 mM). Moreover, the macromolecular solute enhancement of fusion yield is most pronounced at very low concentrations. Raising the concentrations higher has little or no effect (Fig. 2). This suggests that some part of the electrofusion phenomenon is very sensitive to small concentrations of these molecules.

Up to now, the relationship between electrofusion yield and the electric field strength and the duration of the fusogenic pulse (for both square and exponentially-decaying waveforms) has been fairly well characterized [2,3,8,9]. Some experimental data has been obtained for the fusion yield dependence on lipid soluble chemicals [10] but reports on the fusion yield dependence on ionic strength are conflicting [2,3,11–13]. For both the rabbit and human erythrocyte ghost membranes in sodium phosphate buffer (pH 8.5) the fusion yields are strongly and approximately proportional to buffer strength in the range of 2 to 20 mM [14]. Also, there is now evidence that it may be possible to experimentally determine the identity of the components or the biologically-relevant properties of biomembranes which determines the fusabilty of the membranes [15]. The degree of resealing of the ghost membranes in the presence of different macromolecules is thought to be relatively unimportant in this study for two reasons. First, rigorous studies have determined that there is only one hemolytic hole per ghost membrane [16,17]. The probability of this hole being present at the contact junctions where fusion will either take place or not take place is thus very low (i.e., proportional to the shared contact area between two adjacent spherical ghosts in a pearl chain divided by the total membrane area per ghost membrane). Second, the exact size of the hole is not important as long as it is smaller than the effective diameter of the macromolecules trapped in the cytoplasmic compartment. Our preparations are washed after the resealing step, and it was previously determined in our laboratory that little or no leakage of macromolecules takes place if they have a diameter larger than about 1.0 nM [18].

This paper is the first to report a strong general effect on electrofusion yield from three very different aqueous soluble macromolecules with similar molecular weights and that the effect depends, for a pulse with a given decay half-time, on a very narrow range in pulse field strength. The basis for this effect is obscure although data from a report from another laboratory [13] on studies of electroporation and electrofusion in CHO cells with macromolecules in the medium can be interpreted in a way that suggests that the molecules occlude or partially occlude the electropores. If this were the case in our experiments, then the occlusion might also help prevent the pulse-induced transmembrane voltage from being as quickly drained off after the pulse and thus promote fusion. Conversely, the presence of macromolecules in the extracellular compartment (as applies to the data for the situation depicted in Fig. 3) may increase the weak membrane attraction forces [19] which would be expected to lead to higher fusion yields. However, in that report the attraction forces uniformly increase while in our data, and with the exception of reproducible fluctuations in fusion yeild in the relative hemoglobin concentration of 0.05, the fusion yields show yields with a generally decreasing trend as the hemoglobin concentration increases.

Another factor which could play a role in our data is the effect from various osmotic pressure differences between the macromolecular concentrations in the cytoplasmic compartment and the absence of macromolecules in the external buffer (Figs. 1 and 2) or the various concentrations in the external buffer and the constant concentration in the cytoplasmic compartment (Fig. 3). The relationship of osmotic pressure to solute concentration is generally accepted to be a smooth natural logarithm function of the ratio of concentrations on each side of a membrane [20], with minor deviations from ideality for real solutions. In contrast, our fusion yield dependences on macromolecular solute concentrations are strong only for low concentrations of hemoglobin (0.07% to 0.26–0.6% in Fig. 2) in the cytoplasmic compartment. Also, the fusion yield has a complex dependence when hemoglobin is present in the cytoplasmic compartment and the external hemoglobin concentration is varied (Fig. 3). This suggests that the relationship between fusion yield and osmotic pressure is either not simple or is not involved in a significant way in the fusion mechanism.

This study was supported by Office of Naval Research grant N00014-89-J-1715. Technical Assistance was by Mrs. Myong-Soon Cho.

Note added in proof: (Received 15 May 1990)

I thank Dr. L.V. Chernomordik for calling my attention to a report of a study of Sendai virus-induced fusion of human erythrocyte ghosts in which fusion could be observed only if the white ghosts were loaded with bovine serum albumin or Dextran [22].

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