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We have previously shown by thin section electron microscopy (BJ 60:1026-1037) that in a contact zone induced between erythrocyte ghosts by dielectrophoresis, the use of an electrofusion protocol converts the contact zone into a fusion zone (FZ) containing from 1-225 fusion pores per μm^2. Also, after creation, both the i) FZ stability, and ii) the time-dependent diameter expansion rates of the fusion zone are strongly dependent on an intact spectrin network. In the present project, and using computer-assisted analysis on video-recorded phase optics images of fusion zone diameters, we have found that the FZ diameter vs. time kinetics has two, or three, distinct phases (I-III) in erythrocyte ghosts with an intact or heat-disrupted (42 oC, 20 min), respectively, spectrin network. These phases, revealed by FZ diameter vs. time measurements under some combinations of various electric pulse parameters, temperatures (4-33 °C) during measurements, heat treatment temperatures (39-50 °C), and dielectrophoretic force (E_{ac} = 3.25,

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4.25, & 5.25 V/mm), and under certain conditions, were remarkably *independent* from one another, yet the durations of Phase I (1.0-1.2 sec) and Phase II (4.0 sec) were remarkably *invariant* regardless of the variable studied. This suggested the existence of a complex but dissectable interplay of biomechanical factors. Our ability to *control* the induction of a fusion zone between two erythrocyte ghosts and to quantitatively follow the post-fusion swelling or rounding-up of a two ghost fusion product, as a model for cell fusion, may allow the measurement, dissection, and discovery of new membrane/spectrin biomechanical properties and cytoskeletal influences as could come into play in, for example, virus-induced syncytium formation. Glycerol, pH, diamide, wheat germ agglutinin, 2,3 diphosphoglycerate, and NEM (a crosslinking agent), and ethanol treatments all had reproducible and very specific effects on the kinetic phases and the fusion product characteristics.

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FINAL REPORT ON ONR GRANT N00014-92-J-1053

PRINCIPAL INVESTIGATOR: Arthur E. Sowers, Ph.D.

GRANTEE: Department of Biophysics, University of Maryland at Baltimore, School of Medicine

GRANT TITLE: Post-fusion Membrane Reorganization

Start Date: 28 OCT 1991

Funding Period: 28 OCT 1991 - JAN 31 1993

RESEARCH OBJECTIVES: To understand how membrane curvature undergoes spontaneous and natural shape changes following membrane fusion and using an electrofusion paradigm.

REPORT:

Background: The appearance of a number of membrane fusion sites (or pores) in a given area shared by two close spaced biomembranes defines a "fusion zone" and has been seen in a number of artificial and natural contexts. Using the electrofusion protocol to induce membrane fusion and erythrocyte ghosts as a model, an early paper from this laboratory (Chernomordik and Sowers, 1991; Biophys. J. 60:1026-1037[1991]) described such membrane fusions in terms of lumen-producing and non lumen-producing fusion products as they were visible by phase optics. Non lumen-producing fusions (termed in this paper as "flat diaphragm" fusions) always appeared in phase optics as a straight black line at the junction between two ghost membranes in contact. A recent paper from this laboratory, not only reported that the number of lumen-producing fusions (= "open lumen" fusions) would be dramatically increased if erythrocyte ghosts were subjected to a heat treatment (42 °C, 10 mins) but also showed that what appeared in phase optics as a flat diaphragm was actually, at the ultrastructure level, a double membrane multiply perforated with fusion sites (or pores). Also, because the heat treatment was within the known calorimetric transition for spectrin, it was hypothesized in Chernomordik and Sowers (1991) that the state of spectrin played a significant role in determining the final morphological fate of fusion induced between two plasma membranes.

This study: The purpose of the present study was to: i) examine the kinetics of the diameter increase in open lumen and flat diaphragm fusion processes with greater spatial and temporal resolution and also over a greater time interval than in the previous work (Chernomordik and Sowers, 1991), ii) characterize the effect on these kinetics from the fusogenic electric pulse, and the strength of the dielectrophoretic forces which were used to cause membranes to align into the so-called pearl chain formation, iii) more fully determine the kinetics of fusion zone expansion

as a function of heat treatments which involve temperatures that progressively cross the known spectrin calorimetric transition, and iv) attempt to make a preliminary qualitative identification of the involved forces.

Quantitative measures of the time dependent increase in the diameter of fusion zones (FZs)(see Fig. 1), induced by an electric pulse between pairs of erythrocyte ghost membranes, as the two small spheres became one large spherical fusion product, were made by computer-assisted video phase optics microscopy. An example is shown in Fig. 2. Ghost membranes were electrofused either: a) with the spectrin network intact (control), thus producing flat diaphragm-type FZs, or b) after a 42 °C, 20 min heat treatment, thus producing open lumen type FZs. FZ diameters for both control and heat-treated ghosts increased in three distinct kinetic phases: i) a rapid (1 sec long) large exponential shift followed by ii) a slow (4 sec long) small but linear increase, followed by iii) a third kinetic phase beginning at 5 sec after fusion which continued the linear increase, but at an even slower rate. In the heat-treated ghost membranes, the rate in the third kinetic phase, and only the third phase, was substantially higher but still essentially constant until the end of the measurement period (120 sec). If the heat treatment crossed a threshold (45 °C) then the rate of the Phase I expansion was dramatically increased and completely spherical fusion products were achieved within about 1 sec instead of more than 1-2 min after the fusogenic pulse (Fig. 3). Conducting the measurement of FZ diameter increase at different temperatures alters primarily the rate of the first kinetic phase, but much less so phase III and not at all phase II. Changing the dielectrophoretic field strength after, but not before, the fusogenic pulse had a marked effect on the rate of open lumen FZ diameter increase almost exclusively in Phase III. While experimental conditions only affected the *rates* of FZ diameter increase in the various kinetic phases, the *time intervals* of all three phases were remarkably invariant. The details of these finding have been submitted for publication.

Additional data and analysis show that our system can be used to elucidate not only much more about the spectrin-cytoskeletal function than originally thought but also the possibility that these functions play significant roles in nucleated cell fusion and cell morphological changes other than during fusion.

Further analysis of the data, which will go into the second of the two part report (Wu and Sowers) will cover much if not most of the data in Table I and as of this writing is in preparation. As this work is still in progress, and knowledge of an extension of additional funding under N00014-92-J-1053 has been made available to us, a no cost extension has been requested in writing, and approved verbally, as of this time. The data suggest that the spectrin system was responsible for two kinetic phases (1-5 sec and 5-120 sec interval): As the 39 - 47 °C spectrin calorimetric transition is crossed, both the 1 - 5 sec linear phase and the 5 - 120 sec phase are eliminated, and the 0 - 1 sec interval increases to the full diameter rather than a fractional increase (Fig. 3). In other words, no flat diaphragm is ever seen and the cell rounds up in one second! The range of the heat treatment is consistent with the known spectrin calorimetric transition. Furthermore, the fact that glycerol protects the kinetic release suggests that the heat treatment effect is more likely due to a protein denaturation than something like a heat induced vesicularization leading to lipid loss from the membrane. These findings are

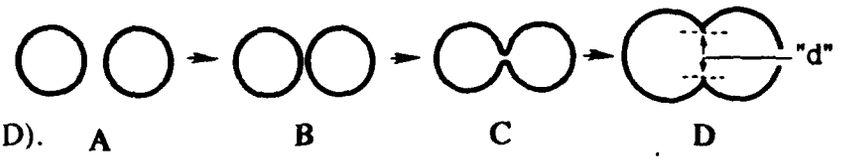

Fig. 1. Fusion of membranes (B → C) is followed by fusion of cells (C → D).

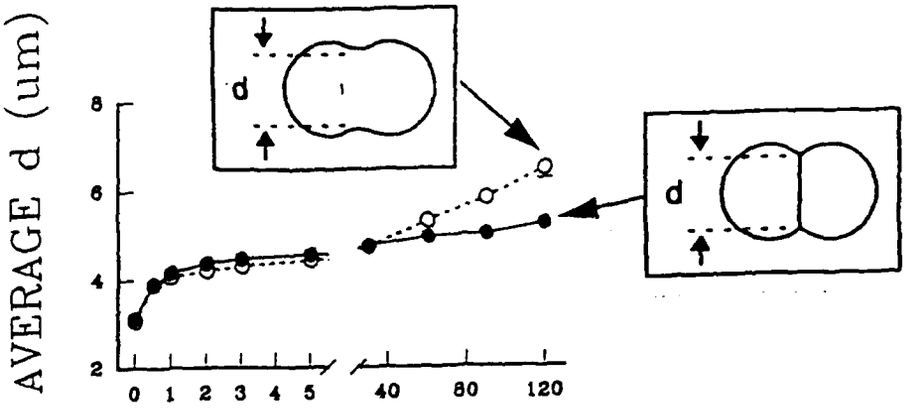

Fig. 2 The fusion zone diameter, d, vs. time relationship. Control ghost membranes (solid circles above) have a rapid, expansion lasting 1 sec followed by a slower expansion (1 to 120 sec). Spectrin network disruption by heat (open circles), causes a third kinetic phase at 5 sec (not noticeable until 30 sec, hence the two different x-axis scales), and leads to a open lumen (cf. insets).

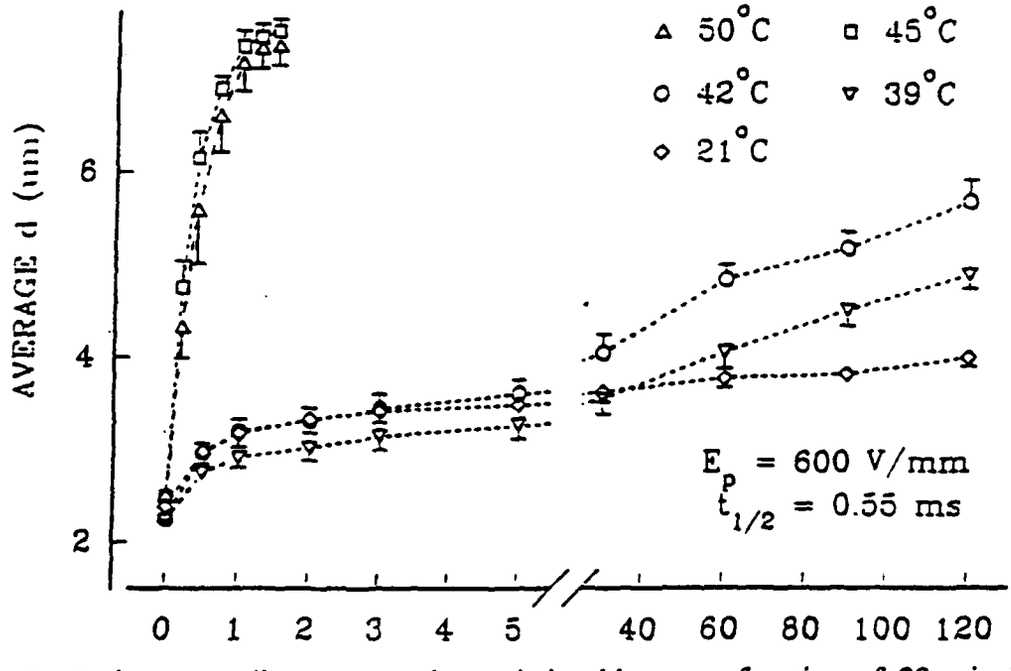

Fig. 3. Fusion zone diameter vs. time relationships as a function of 20 min heat treatments at temperatures given. All led to open lumens except for the 21 °C.

consistent with a spectrin-membrane system restraining substantially any cell morphological changes.

The first two of the three kinetic phases we found were unusual for two reasons. First, both Phase I and Phase II had constant time intervals regardless of the chemical or physical conditions (including temperature). Viscoelastic properties of materials are normally linear with physical dimensions. However, the biomembrane phases is *time* dependent rather than *dimensionally* dependent. Second, the general effect of the heat treatment was to *increase* the rate of change even though many papers showed that a heat treatment caused a *decrease* in erythrocyte deformability, as measured by techniques different than ours. The second is a paradox which implies that there is a fundamental and significant gap in our understanding of spectrin-cytoskeletal function.

Table I is a reproduction of Table 14, page 137 of Yankuan Wu's Ph.D. dissertation (partly supported by the grant), and is a summary which not only includes the effects also described in the original proposal. This data are significant because they show that there are two new classes of observable characteristics (right two columns of table) and significant but specific effects on those observables from ten additional chemical and biochemical factors (items 8-17 in table):

A total of five objective characteristics exist for the description of the morpholigical development of fusion zone. These include not only the three original kinetic characteristics, but also the flat diaphragm lifetime, and the fusion zone population heterogeniety. Showing all graphical data and associated protocols, as well as providing text material, would require at least 30 pages and could not be assembled in time for this report.

Although it was not noticed earlier, additional frame-by-frame playback of video tapes shows that a flat diaphragm is present in all fusion zones for a specific time interval before leading, in a self-completing process, to an open lumen fusion product. Our original observations were concerned with whether or not a flat diaphragm or an open lumen existed at the end of the 120 sec period. What is new about this flat diaphragm "lifetime" is that the flat diaphragm lifetime, itself, is also a variable which is sensitive to physical and chemical factors. Moreover, we found heterogeneity in under some conditions (i.e. flat diaphragm and open lumen products are present simultaneously rather than as one or the other). Note in Table I that for all five colums (the three kinetic components) and the two ("ratio" and "lifetime") structural components, a given factor has specific patterns of influence on an observable characteristics. Also, strong and specific effects (table I) are correlated with several specific treatments which involved diamide, N-ethylmaleimide (NEM), and wheat-germ agglutinin (WGA). These are classic, specific, well characterized, and independent chemical modifiers which have been extensively used to understand the cell biology of erythrocyte shape change and membrane structure-function relationships. Moreover, 2,3-Diphosphoglycerate (2,3-DPG), a naturally occuring metabolite which is known to have a regulatory role in spectrin-cytoskeletal relationships, affects Phase III and only Phase III kinetics (see factor # 14 in table). This means that this system, which shows the five observables each of which have specific sensitivities to a few of at least 17 factors, is likely to permit the elucidation of the function of specific components of the membrane-spectrin

TABLE I

Factors	Figure	Table	Fusion zone kinetics			Ratio: OL/FZ	Flat diaphragm lifetime
			I	II	III		
E_{dc}	18	1	↑			N/A	
$t_{1/2}$	19	N/A	↑			N/A	N/A
$E_{ac.1}$	20	2				N/A	
$E_{ac.2}$	21	3		↑↑	↑↑	N/A	↓↓
$T_{assv.}$	22	4	↑		↑↑	N/A	↓↓
t_{HT}	23	5			↑	N/A	↓↓↓
T_{HT}	24	N/A	↑↑↑		↑	N/A	↓↓↓
pH	26a	6	↑		↑	+++	↓↓↓
	26b	6			↑	+++	↓↓↓
buffer strength	27	7				N/A	
buffer components	28	8	↑↑		↑	+++	↓↓
Glycerol (%)	29	N/A	↑↑		↑↑↑	+++	↓↓↓
Glycerol (method)	30	9			↑	+++	↓↓
Diamide	31	10	↑			+++	↓↓↓
2,3-DPG	32	N/A			↑↑	N/A	N/A
WGA	33	11			.	++	↓↓
NEM	34	N/A	↑↑↑		↑↑	N/A	↓↓↓
EtOH	35a	12			↑	+++	
	35b	12			↑	+++	↓
	35c	12			↑		

Note: t: time. T: temperature. OL: open lumen. FZ: all fusion zone. blanks: no effect or no change.
↑, increase; +, change; ↓, decrease. Increase, change, or decrease is in proportion to the number of symbols.

network. The significance of these findings is that these characteristics can be used as "handles" in completely new experiments to unravel protein-membrane mechanical function! For example, much of the older protein biochemistry literature on isolated spectrin and spectrin related proteins report such characterizations as ion binding sites, isoelectric points, and ionic strength precipitations. The combination of these facts and our variables is likely to permit the assignment of kinetic and structural function to a protein's identity. Also, since spectrin is accepted to be present in all non-erythroid eukaryotic cells, it is expected that our work will have broad significance for cells other than erythrocytes.

Books edited during funding period:

Chang, D.C., Chassy, B.M., Saunders, J.A., Sowers, A.E., eds. (1992) Guide to Electroporation and Electrofusion, (Academic Press) 581 pp.

Allen, M.J., Cleary, S.F., Sowers, A.E., and Shillady, D.D., eds. (1992) Charge and Field Effects-3, (Birkhausen, New York). 502 pp.

Publications during funding period:

Abidor, I.G., and Sowers, A.E. (1992) Kinetics and Mechanism of Cell Membrane Electrofusion. Biophys. J. 61, 1557-1569.

Abidor, I.G., Li, L.-H., and Sowers, A.E. (submitted) Pore Resealing in Electrohemolysis and Electrofusion: Why do Electrofused Erythrocytes Undergo Less Hemolysis than Electroporated Erythrocytes? Biochim. Biophys. Acta

Abidor, I.G., Li, L.-H., and Sowers, A.E. (submitted) Erythrocyte Ghost Electrofusion Yield is Not Different in Subpopulations Fractionated by Osmotic Fragility or Sensitivity to Electroporation. Biochem. Biophys. Acta

Wu, Y.-K., Sjodin, R.A., and Sowers, A.E. (revised and resubmitted) Evidence that Spectrin is Involved in Two of Three Distinct Mechanical Relaxation Components in Pairs of Erythrocyte Ghosts Undergoing Fusion. Biophys. J.

Wu, Y.-K., and Sowers, A.E. (in preparation) Five new kinetic relaxation characteristics in erythrocyte ghost membranes undergoing fusion, and their dependencies on chemical environments.

Book chapters during funding period:

Sowers, A.E. (1992) Mechanisms of Electroporation and Electrofusion. In: Guide to Electroporation and Electrofusion (D.C. Chang, B.M. Chassy, J.A. Saunders, and A.E. Sowers, eds.) Academic Press, San Diego. 119-138.

Chang, D.E., Saunders, J.A., Chassy, B.M., and Sowers, A.E. (1992) An Overview on Electroporation and Electrofusion. In: Guide to Electroporation and Electrofusion (D.C.

Chang, B.M. Chassy, J.A. Saunders, and A.E. Sowers, eds.) Academic Press, San Diego. 1-6.

Chassy, B.M., Saunders, J.A., and Sowers, A.E. (1992) Pulse Generators for Electrofusion and Electroporation. In: Guide to Electroporation and Electrofusion (D.C. Chang, B . M . Chassy, J.A. Saunders, and A.E. Sowers, eds.) Academic Press, San Diego. 555-569.

Sowers, A.E. (1992) The Role of Both Post-Fusion Lumen Expansion and Membrane Fusion in the Entry of Cells by Enveloped Viruses. In: Advances in Membrane Fluidity series, Vol 6 - Membrane Interactions of Human Immunodeficiency Virus (R.C. Aloia, C.C. Curtain, and L.M. Gordon, eds) John Wiley & Sons, Inc. 203-214.

Sowers, A.E. (1993-in pages) Membrane Electrofusion: A New Paradigm for the Study of Membrane Fusion Mechanisms. In: Methods in Enzymology, vol. 220 (N. Duzgunes,ed.)

Abidor, I.G., and Sowers, A.E. (1992) The Coaxial-Pore Mechanism of Cell Membrane Electrofusion: Theory and Experiment. In: Int. Symposium on Charge and Fields in Biosystems (Richmond, 1991)(M.J. Allen, S.F. Cleary, A.E. Sowers, and D.D. Shillady, eds) Birkhausen, New York. 375-409.

Abstracts during funding period:

Abidor, I.G., and Sowers, A.E. (1992) Membrane Electrofusion: Kinetics and Mechanism, Biophys. J. 61: A497.

Wu, Y., Sjodin, R.A., and Sowers, A.E. (1992) A New Approach to the Analysis of Syncytium Formation which Accounts for Spectrin Involvment and Uses Erythrocyte Ghosts and Nucleated Cells as Models, Molecular Biology of the Cell (suppl) 3:269a.

Wu, Y., Sjodin, R.A.,Foster, K., and Sowers, A.E. (1993) What Happens to a Fusion Zone after Fusion Pores are Created in It? Biophys. J. (submitted)

Sowers, A.E., Wu, Y., Lewis, G.K., and Sjodin, R.A. (1993) Multiple, Spectrin-Related but Independent Mechanical Relaxation Components in Erythrocyte Ghosts Undergoing Fusion. Biophys. J. (submitted).