Biochimica et Biophysica Acta 903 (1987) 89–95
Elsevier

BBA 73661

Changes in myoblast membrane electrical properties during cell-cell adhesion and fusion in vitro

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(Received 2 March 1987)

Key words: Conductivity; Radiowave frequencies; Myogenesis; Cell-cell recognition; Myoblast fusion; Cell adhesion; Differentiation; (Chicken myoblast)

Characteristic of the process of myogenesis are the changes in the composition and organization of the cell membrane. While poorly understood, these changes have biochemical and biophysical relevance. Recently, changes in molecular order of the myoblast membrane which accompany differentiation in vitro have been observed (Santini, M.T., Indovina, P.L. and Hausman, R.E. (1987) Biochim. Biophys. Acta 896, 19–25). To further investigate these cell fusion processes we have examined additional physical parameters: conductivity and permittivity of the myoblast membrane during differentiation which reflect the molecular arrangement of the membrane. The determination of these parameters is possible because in the radio frequency range suspensions of cells in an electrolyte buffer show a characteristic conductivity dispersion due to the interfacial polarization. An analysis of our experimental data based on a 'single-shell' model showed that conductivity and permittivity of the membrane of pre- and post-fusion myoblasts varied significantly and abruptly. The conductivity of the cell interior (cytosol) remained constant. We discuss the significance of the observed changes in these membrane parameters for myogenesis.

Introduction

Myogenesis in vivo involves a series of specific cell–cell interactions which culminate in the fusion of embryonic myoblasts into myotubes [1,2]. Myoblast aggregate cultures are a useful model system for studying such interactions [3,4]. When gyrotory rotated, suspensions of embryonic avian myoblasts will form aggregates by 15 h of culture. Cells within these aggregates remain separate until a differentiation-dependent change in cell adhesion which occurs between 33 and 36 h [5]. Subsequently, cells begin to fuse into a syncytium and by 70 h, myoblast fusion is essentially complete. At this time, the entire aggregate constitutes a myoball [3]. Two cell membrane differentiation events: prostaglandin synthesis and its binding to a cell membrane receptor and the characteristic change in cell-cell adhesion, appear to be important in the process which leads to fusion [5,6]. Recently, electron paramagnetic resonance (EPR) studies have demonstrated marked changes in membrane order which correlate with these differ-

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entiation events at the cell membrane [7]. Currently, the biochemical and biophysical bases of these changes in membrane order are not known. However, they may well involve changes in the passive electrical properties (permittivity and conductivity) of the cell membrane.

Hanai and co-workers [8,9] have described a procedure which allows the electrical parameters of a cell membrane to be determined. This involves fitting the general equations of the Maxwell-Wagner effect to the measured cell suspension conductivity as a function of frequency. This technique takes advantage of the fact that suspensions of cells in an electrolyte buffer show characteristic conductivity dispersions (β-dispersions) usually occurring in the radio frequency range. These dispersions are due to interfacial polarization phenomena consequent to a spatial rearrangement of electrical charges adjacent to the surface of the individual cells. This rearrangement is governed by the bulk properties (conductivity and permittivity) of the media of the system. The shell surrounding the cells (the cell membrane) separating the internal and external compartments of the system, enhances the characteristics of the dispersion allowing evaluation of membrane parameters. Therefore, the method can provide useful information about the electrical properties of cells: permittivity and conductivity of the cell membrane and conductivity of the cytosol.

In order to apply this technique to differentiating embryonic myoblasts and myoblast aggregates, a realistic dielectric cell model is necessary. In this paper, an appropriate dielectric model is employed and the electrical properties of the myoblasts evaluated. These are used to help understand differentiation of the myoblast membrane during myogenesis in vitro.

Materials and Methods

Materials. Trypsin was obtained from Difco, soybean trypsin inhibitor and DNAase from Sigma, Dulbecco's Modified Eagle's Medium and penicillin/streptomycin from Gibco Europe, fetal bovine serum from Flow. All other chemicals were obtained from Farmitalia Carlo Erba.

Myoblast cell cultures. Myoblasts were obtained from pectoral muscles of 11-day embryonic chicks and aggregate cultures prepared as described previously [4,7]. The suspended myoblast aggregates were collected for measurements of size (legend to Fig. 2) or of electrical properties (see below).

Conductivity measurements. Myoblasts ($25 \cdot 10^7$) were collected, centrifuged and washed (three times) and resuspended in 1.5 ml phosphate-buffered saline (pH 7.4). Conductivity measurements were carried out in the frequency range from 10 kHz to 100 MHz by means of two impedance analyzers: Hewlett-Packard model 4192A (frequency range 10 kHz to 10 MHz) and model 4193A (frequency range 0.5 MHz to 100 MHz). Both were controlled by a Digital Equipment (DEC) computer system. The conductivity cell is described in detail elsewhere [10]. It consisted of a section of cylindrical waveguide excited well beyond its cut-off frequecy mode. Cell constants were determined by calibration with standard liquids of known conductivity according to Bottomley [11]. The measurements were carried out at a temperature of 30 °C maintained within 0.1 °C. Errors in conductivity were estimated within 1% across the frequency range. Permittivity was calculated from the measurements as described below.

Fractional volume. The fractional volume (Φ) for each sample was determined following measurements of conductivity by determining the volume of the particle suspension (cell plus buffer), then centrifuging the suspension (5000 rpm, 10 min) and measuring the volume of the buffer alone.

Results

Conductivity dispersions

Fig. 1 shows electrical conductivity as a function of frequency for particle suspensions of myoblasts, before (curve b) and after (curve a) membrane fusion. The conductivity of the buffer solution is also shown (curve c). A marked electrode polarization effect exists in the low frequency range (up to $3 \cdot 10^4$ Hz) due to the high values of the d.c. ionic conductivity of these samples (data not shown). This phenomenon is related to the existence of an electrical double layer at the electrode-buffer interface that behaves as a capacitor dependent mainly on the frequency of the applied

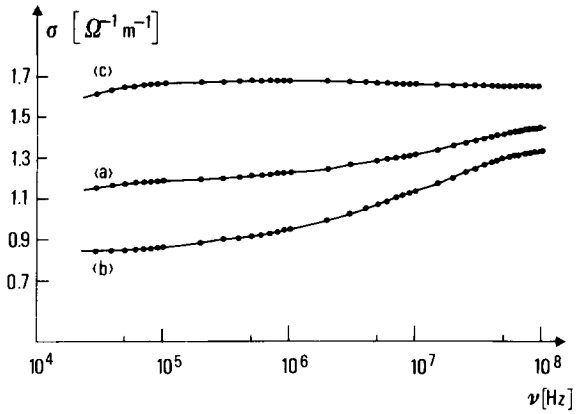

Fig 1. Conductivity of suspensions at 30 °C as a function of frequency: (a) myoballs (90 h) with a fractional volume (Φ) of 0.65, (b) myoblast aggregates (22 h) with a fractional volume of 0.70, (c) phosphate-buffered saline (pH 7.4). The solid lines are drawn for visual purposes only. The frequency dependence of conductivity (at frequencies below 10^4 Hz) due to electrode polarization is not shown.

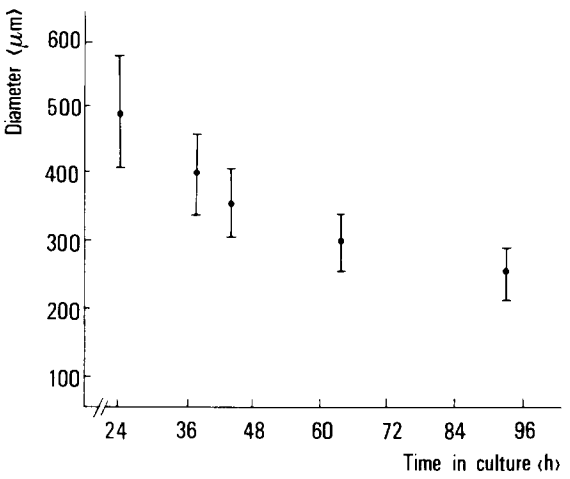

Fig. 2. Average diameters of chick embryo myoblast aggregates with time in culture. Fifteen aggregates were isolated at 24 h of culture and placed in one well of a Linbro multi-well culture plate. At the time specified, each aggregate was photographed. Sterile conditions were maintained throughout the culture period. Each time point represents the mean and standard deviation of the 15 aggregates.

field, buffer conductivity and electrode surface area. This capacitance-like behavior is responsible for the lowering of the measured conductivity values at low frequencies.

Curve c shows that the conductivity of the buffer solution is constant in the range between 10^5 and 10^8 Hz. In contrast, curves a and b show the marked conductivity dispersion typical of interfacial polarization in heterogeneous systems. This occurs at the membrane surface which is exposed to the cell interior (cytosol) or to the buffer solution. The conductivity increment (from low to high frequencies) was less pronounced in curve a (after fusion). This indicated a decrease in the total surface of the membrane. This was supported by the data (Fig. 2) which show that the average diameter (hence membrane surface area) decreased with time in culture.

Dielectric model

The dielectric model adapted here considers the particles (myoblasts and myoballs) as shelled spheres randomly dispersed in a homogeneous medium (Fig. 3). Each particle is considered as a conducting system covered with a less conducting membrane. More complex models, such as the 'double-shell' model of Irimajiri et al. [12], have been considered. However, the consistency of our experimental results with the 'single-shell' model made us confident in its usefulness in modeling myoblast interactions.

The electrical properties of each component of the system are described by the complex conduc-

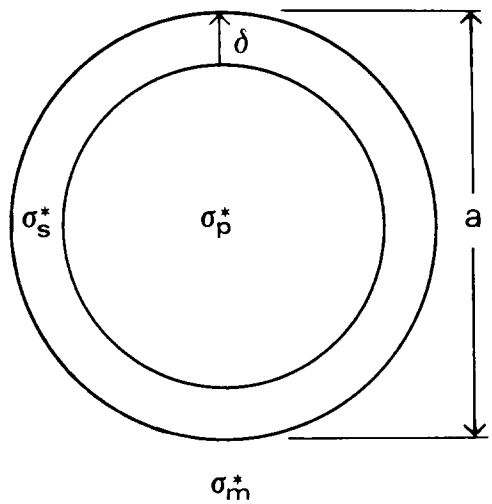

Fig. 3. Geometrical representation of the particles (myoblasts and myoballs) in suspension. Cross-section of a sphere of diameter a and shell thickness δ, σ_m^* is the complex conductivity of the buffer solution (medium), σ_p^* the complex conductivity of the cell interior (cytosol) and σ_s^* the complex conductivity of the cell membrane.

tivities (consisting of a real and an imaginary part) described by the following expressions:

$$\begin{aligned}\sigma_m^* &= \sigma_m + i\omega\varepsilon_0\varepsilon_m \\ \sigma_p^* &= \sigma_p + i\omega\varepsilon_0\varepsilon_p \\ \sigma_s^* &= \sigma_s + i\omega\varepsilon_0\varepsilon_s\end{aligned} \tag{1}$$

where the subscripts m, p and s refer to the buffer solution, cell interior (cytosol) and cell membrane, respectively. ε_0 is the permittivity of free space, ω the angular frequency of the applied field and i the square root of -1. In this case, the real and imaginary parts of the complex conductivities are assumed to be independent of frequency as the dipolar losses due to orientational relaxation phenomena of the bulk media generally occur at microwave frequencies [13]. On the other hand, diffusional motions of lipids and proteins generally contribute to polarization effects [14], but these effects occur at frequencies below those investigated here.

Assuming that the interactions between the suspended particles (myoblasts and myoballs) were negligible [15], the following Maxwell-Wagner equation is applicable

$$\begin{aligned}\frac{\sigma^* - \sigma_m^*}{\sigma^* + 2\sigma_m^*} = \Phi\Big\{&(\sigma_m^* - \sigma_s^*)(2\sigma_s^* + \sigma_p^*) + (\sigma_m^* + 2\sigma_s^*) \\ &\times(\sigma_s^* - \sigma_p^*)\left(1 + \frac{2\delta}{a}\right)^{-3}\Big\}\Big\{(2\sigma_m^* + \sigma_s^*) \\ &\times(2\sigma_s^* + \sigma_p^*) + 2(\sigma_m^* - \sigma_s^*)(\sigma_s^* - \sigma_p^*) \\ &\times\left(1 + \frac{2\delta}{a}\right)^{-3}\Big\}^{-1}\end{aligned} \tag{2}$$

where Φ is the fractional volume of particles of diameter a and shell thickness δ (Fig. 3).

The diameter used for each time point was obtained by microscopic examination of the samples (Fig. 2). For samples up to 50 h, the agregates were treated as collections of single myoblasts with an average diameter of 7 μm [4] as discussed below. Samples beyond 50 h (fused myoblasts) were considered as having a continuous external membrane with the average diameters shown in Fig. 2. In all cases, the value of δ, the thickness of the cell membrane, was assumed to be 7.5 nm [4].

Eqs. 1 and 2 depend on six parameters: conductivity and permittivity of the external medium, of the membrane and of the cell interior (cytosol). The conductivity of the external medium σ_m was measured directly and found to be 1.67 $\Omega^{-1} \cdot m^{-1}$ at 30 °C. The permittivity ε_m was estimated on the basis of the dielectric decrement of a 0.15 M NaCl electrolyte solution [13]. The other four parameters σ_s, ε_s, σ_p and ε_p were obtained by fitting the real part of Eqn. 2 to the experimental conductivity data as outlined by Asami et al. [8,9]. In addition, fitting uncertainties for the four parameters were determined. While the uncertainties of σ_s, ε_s and σ_p were within 5%, an uncertainty of about 20% was found for ε_p.

To validate the fitting procedure used, we examined the effect that changes in the less certain parameters (ε_m which was estimated and ε_p with its high uncertainty) would have on the other parameters. This analysis indicated that ε_m and ε_p have little influence (a few per cent) on the other parameters. For this reason, we were confident of the validity of our values for σ_s, ε_s and σ_p and they will be presented and discussed.

The 'singe-shell' model described here has been successfully used for the analysis of β-dispersions in erythrocytes [16]. Other authors [12,17] have used a 'double-shell' model to justify their experimental data obtained from more complex cells. The present results from myoblasts did not necessitate the use of the more complex model. We treated both cells and myoballs as an external membrane enclosing a cytosol (including internal membranes, organelles and nuclei) with an average conductivity σ_p. The internal structures had a negligible interfacial polarization as they are immersed in the same internal medium (cytosol).

Application to the myoblasts

The multistep process by which embryonic chick myoblasts adhere to one another and fuse occurs over the culture period from 33 to about 70 h. Thus, we chose to initially investigate the values of the membrane parameters of myoblast aggregates before the change in cell adhesion and after fusion was essentially complete.

As noted above, the values for the electrical parameters depend on the geometric model used to describe the particles. The myoball, which results from complete cell to cell fusion by 90 h of

culture [4], was treated as an external membrane of 7.5 nm thickness enclosing the internal cell medium (cytosol) with an average conductivity σ_p. Eqn. 2 yielded a membrane conductivity of 10^{-4} $\Omega^{-1} \cdot m^{-1}$, an internal conductivity of 1.2 $\Omega^{-1} \cdot m^{-1}$ and a membrane permittivity of 2.5. Essentially identical values for these parameters were obtained from myoballs at 70 h of culture, just after cell fusion is complete (Figs. 4, 5 and 6).

If the same geometric assumptions were applied to the aggregates of myoblasts before fusion had occurred (20 and 40 h), the observed data could not be accounted for. Not surprisingly, if the aggregates of myoblasts were treated as a single cellular entity of the size observed (Fig. 3), unrealistic values for the electrical parameters were obtained. However, if the aggregates were considered as being composed of individual cells (spheres) with a diameter of about 7 μm, meaningful electrical parameters could be determined. Eqn. 2 now yielded values of $4 \cdot 10^{-3}$ $\Omega^{-1} \cdot m^{-1}$ for the conductivity and about 15 for the permittivity of the cell membranes. The conductivity of the cell interior (cytosol) remained the same as that seen for the post-fusion myoball, about 1.2 $\Omega^{-1} \cdot m^{-1}$.

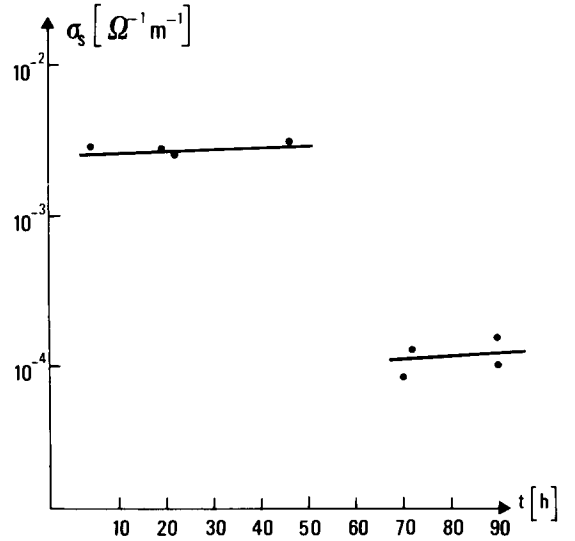

Fig. 4. Conductivity (σ_s) of myoblast or myoball membranes as a function of time in culture. Each point represents the value obtained from the fitting of the β-dispersion curve of Fig. 1 as described in the text. Measurements repeated at or about the same culture time indicate the high reproducibility.

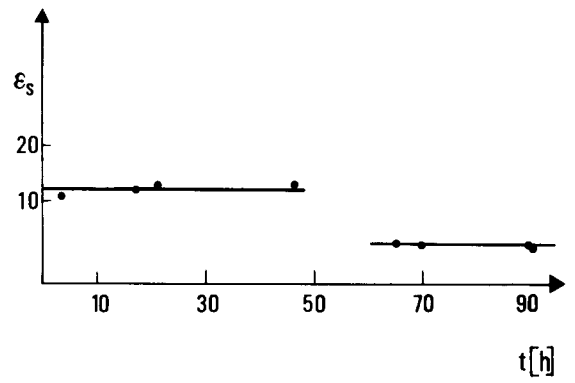

Fig. 5. Permittivity (ε_s) of myoblast or myoball membranes as a function of time in culture. Values were obtained as described in the legend to Fig. 4.

The validity of this treatment of the myoblast aggregate as an assemblage of individual cells was supported by its successful application to the suspension of cells before gyrotory aggregation. Measurements on the suspension of myoblasts after 4 h of culture (before large aggregates had formed) yielded electrical values identical to those obtained from the large pre-fusion aggregates. This confirmed that the myoblast aggregates behaved electrically as the sum of the single myoblast cells where each cell membrane maintains its individuality.

Thus, the conductivity of the myoblast cell membrane remained constant from the beginning of culture until at least 45 h. Between 45 and 70 h of culture, there was a significant abrupt decrease

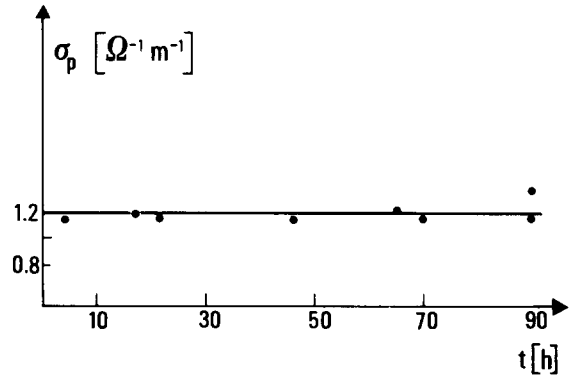

Fig. 6. Conductivity (σ_p) of the cell interior (cytosol) as a function of time in culture. Values were obtained as described in the legend to Fig. 4.

in both membrane conductivity and permittivity (Figs. 4 and 5). In contrast, the conductivity of the cell interior (cytosol) did not appear to change during this period of myoblast differentiation (Fig. 6).

Discussion

The membrane permittivity ε_s and the membrane conductivity σ_s reflect specific structural properties of the cell membrane. The parameter ε_s is related to the distribution of charges and/or polar groups across the lipid bilayer. The conductivity, σ_s, takes into account the different ion transport mechanisms occurring across the cell membrane. The abrupt changes in these parameters between 45 and 70 h of culture suggest a molecular rearrangement within the membrane during that time with a consequent decrease in continuous ion transport. It is important to note that the membrane values approached those of a pure lipid bilayer [18], suggesting that during the fusion process there was a membrane rearrangement which resulted in a more compact structure with reduced fluidity. This hypothesis is also supported by the change in membrane permittivity ε_s which is probably due to a substantial decrease in protein content during myogenesis [19,20].

The lack of change in these same membrane electrical properties over the earlier (0–45 h) period of culture contrasts with the significant changes in myoblast membrane order previously observed with EPR. That analysis showed a peak of membrane order at 12–15 h associated with gyrotory aggregation, and a second peak of membrane order at 38 h associated with the change in myoblast cell-cell adhesion [5,7]. The second peak of membrane order appears to be associated with changes at the external surface of the myoblast membrane [7]. Thus, the processes of gyrotory aggregation of myoblasts and the differentiation events at the cell membrane associated with prostaglandin binding to its receptor and the consequent rapid change in cell adhesion [5] appear to have little effect on the electrical properties of the myoblast membrane. This suggests that the changes in membrane order reflect more subtle interactions between myoblast membrane proteins or lipids which do not change the overall electrical properties of the membrane. Many of the known membrane antigenic changes [21–25] are thought to occur early in myogenesis perhaps concurrently with the changes in myoblast membrane order. Thus, they would occur concurrently with the two changes in myoblast membrane order (the characteristic binding of prostaglandin and its associated change in cell adhesion) before 40 h of culture.

In contrast, the electrical properties of the myoblast cell membrane appear to change later in differentiation, at the time of membrane fusion (between 45 and 70 h). They appear to reflect biochemical changes which correlate with the process of membrane fusion. Significant changes in membrane lipid–protein interactions are known to accompany the process of myoblast adhesion and fusion [26–29]. It is possible that these changes in lipid-protein interaction may occur just before or simultaneously with the membrane fusion event, at the time we observed the change in membrane electrical properties. However, the lack of any change in molecular order over the 40 to 70 h period of culture as measured by EPR (Santini et al., in preparation) makes this unlikely. Instead, the present results suggest that any changes in lipid-protein interaction either occur earlier or are below the resolution of EPR. For this reason, we favor an explanation of the change in electrical properties based on specific membrane proteins. There is substantial evidence that differentiation of the myogenic membrane involves changes in cell membrane channels [30–33]. The changes in membrane conductivity and permittivity observed here might reflect changes in ion channel composition of the differentiating muscle cell membrane or changes in the electrical properties of pre-existing channels.

While fusion in myoblast aggregates is more synchronous than in standard stationary cultures, it still occurs at different places within a single aggregate over a 12 to 15 h period [4]. The finding that a clearly detectable change in membrane electrical properties is associated with fusion might allow a better handle on the timing of the process. This is a necessary step in further understanding the process of actual membrane union. Finally, if this change in electrical properties is associated with biochemical modifications of membrane proteins (possibly ion channels), it will provide us the

means to assay subsequent experimental manipulations. Realization of either of these possibilities requires the extrapolation of the present approach to the period of myogenesis in vitro between 45 and 70 h. That work is currently in progress.

Acknowledgments

We thank Mr. Giacomo Monteleone for assitance with the study of aggregate and myoball size. This investigation was partially supported by Basic Research Grant 1-859 from the March of Dimes Birth Defects Foundation (to R.E.H.) and also by an Italian Consiglio Nazionale delle Ricerche Grant 85.00.304.02 (to P.L.I.).

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