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Cesium ions delay membrane fusion of chick embryo myoblasts in vitro: a conductivity study

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Cesium has a wide range of effects on biological systems. However, the effects of this ion on muscle differentiation are not known. We have recently demonstrated that there is a sharp decrease in the conductivity and permittivity of the membranes of chick embryo myoblasts at the time of fusion (Bonincontro, A., Cametti, C., Hausman, R.E., Indovina, P.L. and Santini, M.T. (1987) Biochim. Biophys. Acta 903, 89–95). Analysis of the conductivity dispersion data in the radiowave frequency range using a 'single-shell' model showed that individual myoblasts and unfused myoballs have significantly higher membrane conductivity and membrane permittivity than fused myoballs. We show here that the sharp fall in these membrane electrical parameters occurs at 60 h of culture and is indeed very abrupt, taking place within one hour. In addition, we also demonstrate that cesium ions delay the sharp decrease in both the conductivity and permittivity of myoblast membranes by about 30 h. We discuss the possible mechanisms by which cesium perturbs potassium transport across these membranes and how this perturbation may affect fusion itself.

Introduction

The fusion of individual mononucleated myoblasts to form multinucleated muscle fibers involves the union of their cell membranes and the subsequent disappearance of these membranes between adjoining cells [1,2]. Myoblasts grown in rotation culture first aggregate and then fuse to form what may be termed 'myoballs' [3]. A series of characteristic developmental events accompanies myoblast fusion including a net calcium influx prior to fusion [4,5], alterations in surface proteins [6,7], changes in the inositol phospholipids [8,9] and the accumulation of acetylcholine receptors as well as acetylcholinesterase [10,11]. In addition, muscle fibers undergo electrophysiological maturation as evidenced by the maintenance of a large resting membrane potential [12], the generation of action potentials and the acquisition of contractile activity [13] with a concomitant increase in sodium channels [14].

We have previously observed that there is a sharp decrease in electrical conductivity and permittivity of the membranes of chick embryo myoblasts grown in rotation culture at the time of fusion [15]. In fact, our analysis using a 'single-shell' model of the interfacial polarization results showed that individual myoblasts and unfused myoballs had a higher membrane conductivity

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than fused myoballs by over an order of magnitude. We interpreted these data as signifying that the sharp decrease in both membrane conductivity and membrane permittivity resulted from changes in ion channel composition of the differentiating muscle cell membrane or changes in the electrical properties of preexisting channels. Thus, the sharp decrease in both electrical parameters observed may be viewed as a decrease in ionic permeability caused by the closing of specific ion channels.

Cesium is not normally present in cells, but, when present, it has a wide variety of effects on biological systems. For instance, cesium is known to become fixed in muscle as was demonstrated with radioactive cesium [16], but the effects of this fixation as well as the effects on muscle differentiation are not clearly understood. As a result, we concentrated our efforts in trying to determine whether the abrupt decrease in conductivity and permittivity of myoblast membranes seen at fusion is perturbed by cesium. Consequently, we added cesium to the myoblast cultures. Our results demonstrate, in fact, that cesium delays myoblast fusion. Cesium ions are also known to interfere with potassium channels in various cell types including muscle cells [17–20]. In light of this, potassium channels may be involved in the electrical changes observed. We discuss the possible mechanisms by which cesium perturbs potassium transport across the myoblast membrane and how this perturbation may affect fusion itself.

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 and fetal bovine serum from Flow. All other chemicals were obtained from Farmitalia Carlo Erba.

Myoblast cell cultures. Myoblasts were obtained from the pectoral muscles of 11-day embryonic chicks and aggregate cultures prepared as described previously [21,22]. For the cesium experiments, 20 mM CsCl dissolved in deionized water was added at 24 h of culture under sterile conditions by which time aggregates had already formed [21]. The flasks were then regassed before returning to incubate. The suspended myoblast aggregates were collected for measurement of size as described previously [15], for determination of percent fusion (legend to Fig. 6) or of electrical properties (see below).

Conductivity measurements. Myoblasts ($25 \cdot 10^7$) were collected, centrifuged, washed three times and resuspended in 1.5 ml phosphate-buffered saline (pH 7.4). Measurements of electrical conductivity were carried out in the frequency range from 10 kHz to 100 MHz by means of two Hewlett-Packard impedance analyzers (models 4192A and 4193A) controlled by a Digital Equipment computer system. The conductivity cell and conductivity measurements are described in more detail elsewhere [23]. Cell constants at the frequencies employed were determined by calibration with standard liquids of known conductivity according to Bottomley [24]. The measurements were carried out at 30 °C maintained within 0.1 C°. Errors in conductivity were estimated to be within 1% throughout the entire frequency range.

Dielectric model. The conductivity data, which display a frequency behavior typical of a β-dispersion, were analyzed using a 'single-shell' dielectric model [25,26]. The model considers the particles (myoblasts and myoballs) as shelled spheres randomly dispersed in a homogeneous medium. Each particle of diameter a is considered as a conducting system covered with a less conducting membrane of thickness δ. For the individual myoblasts, an average diameter of 7 μm was used while for the myoballs, the diameter used for each time point was obtained by microscopic examination. For both myoblasts and myoballs, the thickness of the cell membrane was taken to be 7.5 nm [21]. Assuming that the interactions between the suspended particles (myoblasts and myoballs) were negligible [27], the conductivity of the cell suspension may be described by the following Maxwell-Wagner equation

$$\frac{\sigma_m^* - \sigma^*}{\sigma^* + 2\sigma_m^*} = \Phi \Big\{ (\sigma_m^* - \sigma_s^*)(2\sigma_s^* + \sigma_p^*) + (\sigma_m^* + 2\sigma_s^*)(\sigma_s^* - \sigma_p^*)\Big(1 + \frac{2\delta}{a}\Big)^{-3} \Big\} \times \Big\{ (2\sigma_m^* + \sigma_s^*)(2\sigma_s^* + \sigma_p^*) + 2(\sigma_m^* - \sigma_s^*)(\sigma_s^* - \sigma_p^*)\Big(1 + \frac{2\delta}{a}\Big)^{-3} \Big\}^{-1} \quad (1)$$

where $\sigma^* = \sigma_j + i\omega\epsilon_0\epsilon_j (j = m, p s)$ is the complex conductivity and the subscripts m, p and s refer to the buffer solution, cell interior (cytosol) and cell membrane, respectively, and where Φ is the fractional volume of the dispersed particles. The fractional volume was determined as described previously [15]. This equation depends on six real parameters: conductivity and permittivity of the external medium (σ_m and ϵ_m), conductivity and permittivity of the cytosol (σ_p and ϵ_p) and conductivity and permittivity of the cell membrane (σ_s and ϵ_s).

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 [28]. The other four parameters σ_p, ϵ_p, σ_s and ϵ_s were obtained by fitting the real part of Eqn. 1 to the experimental conductivity data as outlined by Asami et al. [25,26]. 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% resulted 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 are confident of the validity of our values for σ_s, ϵ_s and σ_p and they will be presented and discussed.

Results

In this paper, we report on additional measurements of electrical conductivity of myoblast cell suspensions in the radiowave frequency range during the time interval in which fusion occurs. These measurements were conducted in order to localize more precisely the actual time of membrane fusion which was previously shown to fall between 45 and 70 h of culture [15]. All membrane conductivity and permittivity data, including a more exhaustive examination of the fusion period, are presented in Fig. 1. The results show that the time of cell fusion is 60 h. Therefore, these measurements demonstrate that the changes in electrical parameters occur in a very short period of time (within one hour) and thus act in a cooperative manner. In addition, the conductivity of the cell interior (cytosol) remained constant at the value of 1.2 $\Omega^{-1} \cdot m^{-1}$ (data not shown) as it did previously [15].

After this more precise analysis of the fusion period, we investigated the effects of cesium ions on myoblast cell fusion. Fig. 2 shows electrical conductivity as a function of frequency for particle suspensions of control myoblasts and of

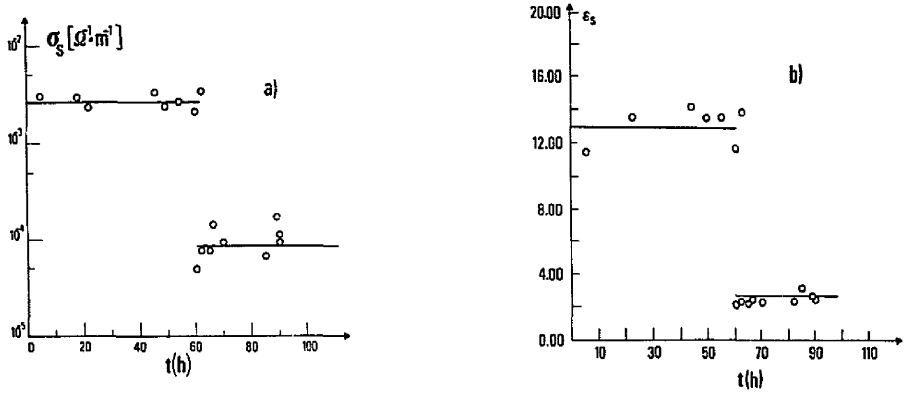

Fig. 1. Conductivity σ_s (a) and permittivity ϵ_s (b) of myoblast or myoball membranes as a function of time in culture. Each point represents the value obtained from the fitting of the β-dispersion curves, examples of which appear in Fig. 2, as described in the text. Measurements repeated at or about the same culture times indicate the high reproducibility. Also, note the abrupt decrease in both σ_s and ϵ_s at 60 h.

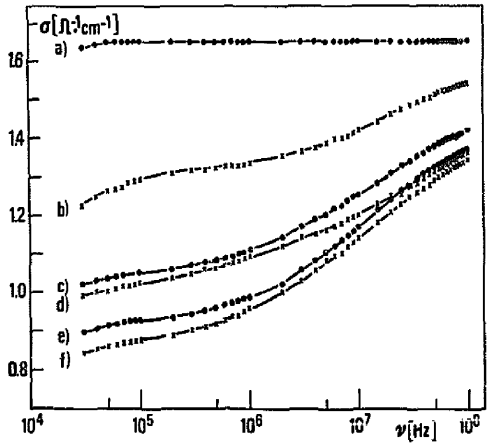

Fig. 2. Conductivity of (a) phosphate-buffered saline (pH 7.4) and (b–f) suspensions of myoblast aggregates or myoballs at 30 °C as a function of frequency. Both normal cultures (●) and cultures to which 20 mM CsCl was added at 24 h of culture time (×) are represented: (b) myoballs (111 h) in CsCl with a fractional volume Φ of 0.63, (c) myoballs (90 h) in normal conditions with a fractional volume of 0.65, (d) myoblast aggregates (90 h) in CsCl with a fractional volume of 0.65, (e) myoblast aggregates (22 h) in normal conditions with a fractional volume of 0.70 and (f) myoblast aggregates (23 h) in CsCl with a fractional volume of 0.64. 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.

myoblasts to which 20 mM CsCl was added at 24 h of culture. Interfacial polarization in heterogeneous systems is a consequence of a spatial rearrangement of electrical charges adjacent to the surfaces separating two media of different electrical characteristics. Each of the curves in Fig. 2 has a different value of conductivity which is proportional to the extent of interfacial polarization present in that system. The buffer solution curve (curve a) obviously shows maximum conductivity independent of frequency. Unfused aggregates (curves d, e and f) have the lowest conductivity at all frequencies since, in these systems, there is a maximum amount of surface (the cell membranes of individual unfused cells separating different cytosols) available for polarization. Finally, fused aggregates (myoballs) have intermediate conductivity values (curves b and c). The overall conductivity behavior is a measure of the surface area available for polarization and, therefore, fusion since this area decreases as fusion proceeds.

Comparison of β-dispersion curves at the same time points with and without cesium permits a qualitative evaluation of the delay upon fusion caused by this ion. In fact, the 90 h curve with cesium (curve d) has, at all frequencies, a conductivity value lower than the 90 h curve without cesium (curve c) indicating that fusion is delayed. In addition, it is important to note that the conductivity increment from low to high frequencies, which can also be assumed to be a measure of the extent of interfacial polarization, varied more slowly upon addition of CsCl. This is supported by the data in Table I which shows that the increments from low to high frequencies obtained by a fitting procedure of the dispersion curves and normalized by the fractional volume Φ of samples with CsCl are delayed with respect to those without the addition of this ion at the same times of culture. It is important to keep in mind that at the time of measurement no cesium ions are present, but rather buffer alone. Therefore, cesium is not affecting the measurements themselves, but rather is affecting biological processes in the differentiating myoblasts in culture before the actual measurements. The delay in fusion is also demonstrated by the aggregate diameter measurements which show a constant value of about 500 ± 40

TABLE I

CHANGE IN CONDUCTIVITY $(\sigma_\infty - \sigma_0/\Phi)$ FROM LOW TO HIGH FREQUENCIES OF MYOBLAST AGGREGATE POPULATIONS TO WHICH CsCl WAS NOT AND WAS ADDED AS A FUNCTION OF TIME IN CULTURE

The conductivity curves were analyzed in terms of a single Debye-type dispersion where σ_0 and σ_∞ are the limiting values at low and high frequency, respectively.

Time in culture (h)	Conductivity change ($\Omega^{-1} \cdot m^{-1}$)	
	without CsCl	with CsCl
20	0.68	0.75
40	0.67	0.75
60	0.65	0.72
80	0.55	0.70
90	0.46	0.65
95	–	0.60
100	–	0.50
105	–	0.35

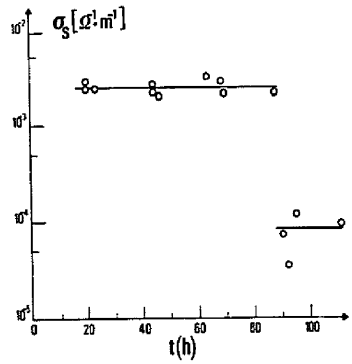

Fig. 3. Conductivity σ_s of myoblast or myoball membranes grown in media to which 20 mM CsCl was added at 24 h of culture as a function of time in culture. Values were obtained as described in the legend to Fig. 1.

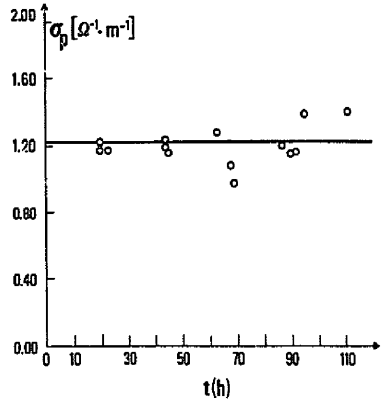

Fig. 5. Conductivity σ_p of the cell interior (cytosol) of myoblast or myoballs grown in media to which 20 mM CsCl was added at 24 h of culture as a function of time in culture. Values were obtained as described in the legend to Fig. 1.

μm from about 24 to 90 h and then begin to decrease, reaching a minimum value of about 380 $\pm$ 30 μm at 125 h. The data are not shown, but were obtained as described previously [15].

Treatment of the dispersion data using Eqn. 1 yielded the results shown in Figs. 3, 4 and 5. Fig. 3 gives the conductivity of the cell membrane σ_s as a function of time in culture. As can be seen, up to 90 h the same conductivity value of about $4 \cdot 10^{-3}$ $\Omega^{-1} \cdot m^{-1}$ is observed. Beyond this time, the membrane conductivity drops abruptly to a value of about 10^{-4} $\Omega^{-1} \cdot m^{-1}$ and remains constant. The time of fusion is shifted about 30 h of culture.

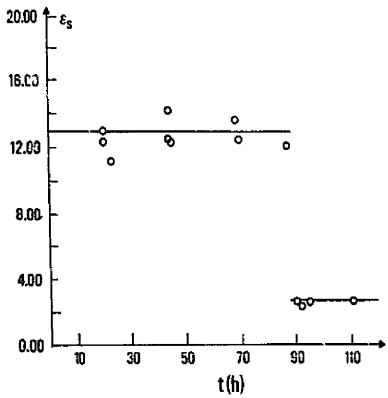

Fig. 4. Permittivity ϵ_s of myoblast or myoball membranes grown in media to which 20 mM CsCl was added at 24 h of culture as a function of time in culture. Values were obtained as described in the legend to Fig. 1.

In addition, the drop in this electrical parameter is also very sharp. Again, fusion seems to occur within about one hour. Permittivity ϵ_s also dropped abruptly at 90 h of culture, falling from a value of about 13 before fusion to one of about 2.5 after fusion (Fig. 4), whereas the internal conductivity σ_p again remained constant at a value of about 1.2 $\Omega^{-1} \cdot m^{-1}$ for the entire culture period (Fig. 5). The conductivity of the cytoplasm may remain constant throughout in vitro myogenesis because the differentiative events taking place in the cell before, during and after fusion, although important biochemically, seem not to influence significantly the high bulk ion concentration of the cytosol and thus the electrical properties measured here. These events may be similar to those taking place in the membrane itself before and after fusion which also do not influence membrane conductivity and permittivity significantly. Only fusion is an event so great and so important on a physical level that, when it occurs, conductivity and permittivity of the membrane vary so dramatically.

Finally, we used an alternative means of establishing the extent of cesium-induced fusion delay. Percent fusion was measured in myoblast aggregates at different culture times in both control cultures (no CsCl added) and cultures to which CsCl was added. The results appear in Fig. 6. As can be seen, in control cultures (a), 90% fusion is

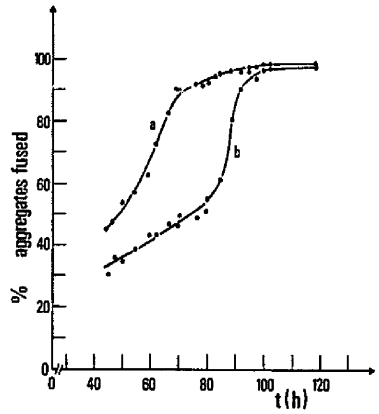

Fig. 6. Percent fusion of chick embryo myoblast aggregates grown in normal culture conditions (a) and in media to which 20 mM CsCl was added at 24 h of culture (b) as a function of time in culture. Light microscopy was used to determine percent fusion by counting the appearance of smooth membrane edges in aggregates. Even if smoothness did not appear along the entire periphery of the aggregate, but was only partially present, the aggregate was considered fused. If no smoothness could be seen, the aggregate was considered unfused. The number of fused aggregates was then divided by the total number of aggregates observed (about 100 for each time-point). In order to ensure reproducibility, three separate series with overlapping time-points were used. The error is ±10 aggregates for each time-point.

complete at about 65 h while in cesium cultures (b), 90% fusion is reached 30 h later, at about 95 h. Due to the procedure used, which considers myoblast aggregates fused even if the fusion process is not complete, an overestimation of per cent fusion, especially in the pre-fusion stages, occurs. For this reason, the curves do not show abrupt changes at the time of fusion.

Discussion

The ionic environment of myoblast membranes changes dramatically at fusion. This was demonstrated by us in a previous report [15] where both membrane conductivity σ_s and membrane permittivity ϵ_s were found to fall substantially at the time of membrane fusion during in vitro myogenic differentiation. Membrane conductivity is a measure of the overall ionic transport across the cell membrane through ion channels while membrane permittivity takes into account the distribution of charges and/or polar groups across that same lipid membrane. Therefore, conductivity may be viewed as a measure of the dynamic processes dependent upon net ionic fluxes whereas permittivity, although indirectly dependent upon these same dynamic processes, is indicative of the static charges present in the membrane. Since both of these parameters vary, both dynamic and static ionic properties of the myoblast membrane seem to be affected by the fusion process. The present experimental results demonstrate that the significant decrease in electrical conductivity and permittivity of the myoblast membrane seen at fusion is abrupt. This drop takes place within one hour and appears to be highly synchronized since the decrease observed is not gradual. This may indicate that the mechanisms which trigger membrane fusion act in a cooperative manner and that membrane fusion itself is an 'all or none phenomena'.

We believe that we are able to observe such abrupt changes in membrane electrical properties because of the culture system used. Aggregate cultures are much more homogeneous and synchronous than stationary muscle cultures [3,21]. In fact, the aggregates and myoballs are composed entirely of myoblasts and exclude fibroblasts which adhere to the air/media interface of the culture flasks. Since the electrophysiological properties of myoballs have been found to be quite similar to those of myotubes [29,30], it is more convenient to use this more homogeneous and synchronous system for these types of studies. Finally, our non-invasive method of measuring the membrane conductivity and membrane permitivity of myoblasts during differentiation, which requires that the cell populations be in suspension, may be more suited to fusion studies than stationary cultures for the following reason. In this system, the aggregates and myoballs grown in this manner are well-adapted to being in suspension and thus undergo no stress during the measurements themselves. Differentiation events are, therefore, seen more clearly.

Cesium ions block potassium conductance through potassium channels in a wide variety of cells [17–20, 31–38]. The mechanism by which cesium blocks potassium conductance is not completely understood and may indeed vary from one type of potassium channel to another. However,

its mode of action in the potassium channel known as the inward rectifier of frog skeletal muscle may serve as an example. Inward-going or anomalous rectification through this channel is characterized by a high conductance for the inward potassium current and a low conductance for the outward potassium current. Inward rectification with similar properties has also been described in starfish eggs [36], in tunicate eggs [37] and guinea pig olfactory cortex neurons [38]. Cesium has been shown to interfere with this anomalous rectification in frog skeletal muscle [19,20], in starfish eggs [17, 36] and in tunicate eggs [37]. In muscle, the block of inward potassium currents is instantaneous, voltage-dependent and increases with hyperpolarization of the cell membrane as well as with the increase of external cesium concentration. The outward potassium current is not affected [20]. In starfish eggs, however, the block is time-dependent and appears to be sensitive to the potassium concentration of the solution bathing the cells. Increasing the external potassium concentration augments the block caused by a particular cesium concentration at a fixed membrane potential [36]. A similar effect of potassium concentration was also found in the block of anomalous rectification by cesium in tunicate eggs [39]. Thus, cesium does not seem to necessarily block potassium conductance completely, but rather appears to interfere with this conductance in one direction only. Consequently, potassium concentration on the opposite sides of the membrane channels blocked by cesium may also be influenced. The cell potassium channels are unable to maintain a proper potassium gradient between the inside and outside of the cell.

The membrane conductivity results presented in this report seem to indicate that cesium is, in fact, interfering with potassium transport across the myoblast membrane and that this perturbation is delaying fusion. But, if this is the case, then what may be the precise role of potassium during myoblast differentiation? Potassium, together with sodium, is primarily responsible in generating the membrane potential of cells. Therefore, potassium may be necessary in helping to generate a particular membrane potential necessary for fusion. In fact, the resting membrane potential was shown to rise during myogenic fusion [12,40]. The resting membrane potential is a measure of the relative ionic environment between the inside and the outside of cells as well as the membrane conductivity of the various ionic species present. Therefore, at any given moment, it too is a consequence of the transport mechanisms across the cell membrane which are responsible for and/or affect membrane conductivity and membrane permittivity. Consequently, it may be expected, as was in fact demonstrated by our results, that membrane conductivity and membrane permittivity are related to resting membrane potential.

Potassium may also be important during myoblast fusion in controlling calcium entry into cells. In fact, calcium entry into myoblasts prior to fusion has been shown to be voltage-dependent, being maximally stimulated by 16 mM potassium [41]. Calcium is known to be essential for membrane fusion during both alignment/aggregation [3,42] and also in actual membrane union [4,5]. Thus, potassium concentration may also be one of the control mechanisms for calcium entry and subsequent membrane fusion. Cesium is thus influencing the concentration of potassium present and, as a consequence, calcium entry may be inhibited resulting in fusion delay. If inward potassium flux is blocked, a higher concentration of potassium than normal would be present outside of myoblasts. In support of this, there is evidence that high concentrations of external potassium inhibit myoblast fusion [43].

The effects of cesium described thus far are sufficient to explain the changes in membrane conductivity which are influenced by active transport mechanisms observed during myogenesis. However, the changes in membrane permittivity, which depend primarily on fixed charges of the membrane, need further clarification. The most straightforward explanation may be that not only is cesium affecting total ionic environment of the myoblast membranes through transport mechanisms and thus permittivity indirectly, but that it is also influencing channel protein structure itself. Cesium may be binding and becoming fixed to potassium channels and thus changing their fixed surface charge. In fact, cesium is believed to occupy potassium binding sites in potassium channels and then to become lodged in the channels unable to move through them because of its large

size and impermeant nature [18]. In addition, cesium may also be affecting the production of myoblast membrane phospholipids and thus total net fixed charge. In fact, preliminary data indicate that changes in phospholipid profile during myogenesis are affected by cesium (Santini et al., in preparation). Specifically, the increase in total phosphatidylcholine of the cell membrane and the decrease in total phosphatidylethanolamine seen at fusion in normal cultures are not seen in cultures to which cesium was added. Different quantities of specific phospholipids and thus different charges are present. Phospholipid type and/or charge can interfere with active transport processes directly. In fact, the type of phospholipid environment in which ion channels are embedded can vary their transport properties [44–46]. Conductance is much higher in negatively charged membranes than in neutral membranes [44,45]. Thus, phospholipid class is important in protein channel conductance. Muscle differentiation may provide for the production of the lipids and proteins necessary for certain developmental events such as fusion. The data presented here seem to indicate that cesium is interfering with these mechanisms.

Acknowledgments

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