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Prostaglandin dependence of membrane order changes during myogenesis in vitro

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Myogenic differentiation in vitro involves at least three events at the cell surface: binding of prostaglandin to cells, cell-cell adhesion, and fusion of the myoblast membranes into syncytia. Previous work has suggested that binding of prostaglandin is causal to the change in cell-cell adhesion and that both are accompanied by a characteristic reorganization of the myoblast membrane detected as a transient increase in membrane order by electron paramagnetic resonance. We show here that this membrane order change, which reaches a maximum at 38 h of development in vitro, was the last membrane order change before bilayer fusion which begins several hours later. This membrane order change, which accompanies the change in cell-cell adhesion, was dependent on the availability of prostaglandin. In myoblasts maintained in indomethacin, where further differentiation is known to be blocked at the prostaglandin binding step, the membrane order change did not occur. However, if myoblasts are provided with exogenous prostaglandin, the membrane order change occurred and differentiation proceeded. The results indicate that the basis of the membrane order change was the reorganization of myoblast membranes to allow increased adhesion and prepare the membrane for bilayer fusion. They also demonstrate that, like the increase in myoblast adhesion, the membrane order change was dependent on prostaglandin being available to bind to its receptor.

Myoblast fusion in vitro creates the syncytium necessary for assembly of the proteins and structures characteristic of mature muscle [1–3]. However, many of the macromolecules characteristic of myogenic differentiation appear before bilayer fusion [4–6]. This has led to increased interest in those aspects of myogenic differentiation which precede fusion of the myoblast membranes [7–12]. Myoblast surface events may play a role in the cell-cell communication necessary to coordinate both the synchronous development of contractile proteins and bilayer fusion [13–14]. Electron paramagnetic resonance (EPR) study of membrane bilayers with spin-labels provides a sensitive means of detecting events in cell membranes. We have described two peaks of membrane order during myogenic differentiation in vitro [15]. The first, coincident with gyrotory aggregation of myoblasts, extends deeply into the membrane bilayer and reached a maximum at 15 h. After a characteristic decrease in membrane order, a second maximum occurs at 38 h. This latter peak of membrane order is associated with binding of prostaglandin to a membrane receptor and a change in cell-cell adhesion [16]. We suggested one of these events caused the 38 h increase in membrane order and suggested that it might be the last

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major membrane reorganization before bilayer fusion [16].

Recent work indicates that the change in cell-cell adhesion and subsequent myogenesis depends on the binding of prostaglandin to its receptor. Inhibition of production of endogenous prostaglandin by the myoblasts blocks adhesion and fusion [16]. Addition of exogenous prostaglandin rescues both these events. Here we ask if the 38 h membrane order change is also dependent on prostaglandin production and if there were additional membrane order changes prior to bilayer fusion. To do this, we extended our measurements of membrane structure by EPR through the period of myoblast fusion in vitro and found no further changes in membrane order. We also showed that the 38 h membrane order peak was sensitive to indomethacin and rescued by prostaglandin. We conclude that the 38 h membrane order change is the last major membrane reorganization before bilayer fusion and that this reorganization is dependent on binding of prostaglandin to its receptor [17].

Trypsin was obtained from Difco, soybean trypsin inhibitor, DNAase, indomethacin [1-(*p*-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid] and prostaglandin E_1 from Sigma, Dulbecco's modified Eagle's medium and penicillin/streptomycin from Gibco Europe, fetal bovine serum from Flow and potassium ferricyanide [$K_3Fe(CN)_6$] from Merck. The spin-labels 5-nitroxystearate (2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl), 12-nitroxystearate (2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxyl), and 16-nitroxystearate (2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl) were purchased from Aldrich. All other chemicals were from Farmitalia Carlo Erba. Myoblasts were obtained from pectoral muscles of 11-day embryonic chicks and aggregate cultures prepared as described previously [14,15]. Additions of indomethacin and prostaglandin to the cultures are described in the figure legends. Myoblasts were prepared and EPR measurements taken as described previously [15].

Studies over the first 48 h of myogenesis in vitro show that the 38 h peak of membrane order is most apparent with the 5-nitroxystearate spin-label which probes the bilayer surface, and is less apparent with the 12-nitroxystearate spin label which probes more deeply into the membrane [15]. To determine if there were further membrane order changes before bilayer fusion, we extended our investigation to 72 h in vitro when fusion is virtually complete [14]. Fusion in these myoblast aggregates begins after 40 h and continues until all nuclei appear within the same cytosol sometime after 60 h [14]. There were no further membrane order changes beyond 38 h detected with these spin labels (Figs. 1 and 2). In addition, testing with the 16-nitroxystearate spin label, which is thought to probe yet more deeply into the membrane [18] showed little change in membrane order throughout myogenesis in vitro (Fig. 3). Thus, the biochemical basis of the 38 h membrane order peak is associated with the surface of the bilayer and it is the last major change in membrane order before fusion. This confirms findings with several techniques that there is a decrease in myoblast membrane organization prior to fusion [1]. Comparison of the absolute values of $2T'_{\parallel}$ for the three spin-labels demonstrated that the outside of the bilayer was more ordered than the inside. Furthermore, the gradient of change in membrane order was greatest between the 5- and 12-nitroxystearate spin-labels, suggesting a steep decline in mem-

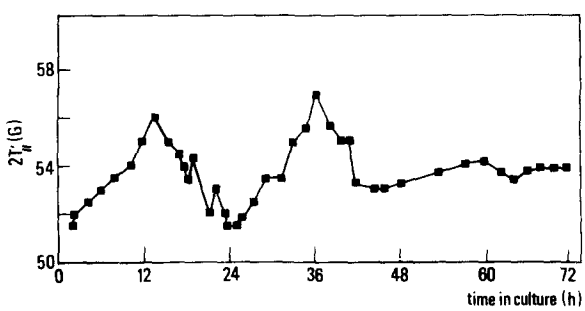

Fig. 1. Myoblast membrane order as represented by $2T'_{\parallel}$ using the 5-nitroxystearate spin-label plotted as a function of time in culture. Bilayer fusion begins in these aggregate cultures after 40 h and continued through 60 h [14]. The plot represents EPR measurements from seven separate experiments with overlapping time points. At each time point, the average value is shown. For each experiment, five or more measurements of $2T'_{\parallel}$ were taken per time-point. The plot from 0 to 48 h represents previously published data [15] plus additional time points. The plot was obtained by connecting the experimental points.

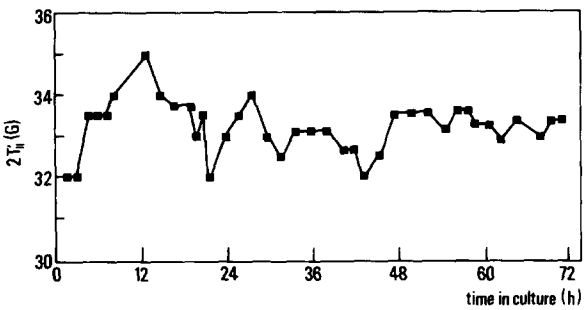

Fig. 2. Myoblast membrane order as represented by $2T_{\parallel}'$ using the 12-nitroxystearate spin-label plotted as a function of time in culture. The plot was obtained as described in Fig. 1.

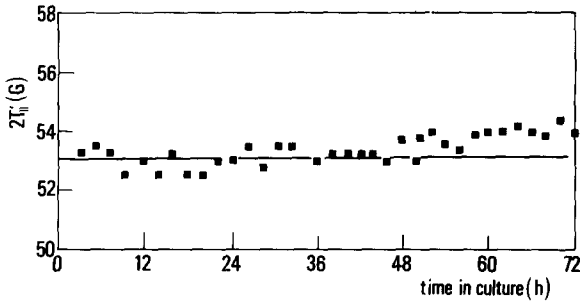

Fig. 4. Myoblast membrane order as represented by $2T_{\parallel}'$ using the 5-nitroxystearate spin-label plotted as a function of time in culture. Indomethacin (30 μM) was added [16] at zero-time in culture. The straight line which best fits the data was plotted.

brane order when moving in from the bilayer surface. Thus, there appear to be no major changes in membrane order within the bilayer interior which were necessary for myoblast fusion. The changes in membrane organization near the bilayer surface which do appear to be necessary occur, at least, 10 h before significant membrane fusion can be detected [14] (Fig. 1).

Previous work showed that gyrotory-mediated aggregation (while a useful tool for bringing the cells into contact) was not necessary for subsequent myogenesis [16]. Thus, to allow for removal of residual prostaglandin, we blocked prostaglandin synthesis by adding indomethacin [16] at the beginning of culture. Membrane order did not change for 27 h (Fig. 4. While the prostaglandin receptor can be detected under these conditions, the change in cell-cell adhesion and fusion do not occur [16]. However, both these events are rescued by addition of prostaglandin E_1. Similarly, the 38 h peak of membrane order was also rescued (Fig. 5. Thus, it appears that this peak of membrane order is uniquely associated with the availability of prostaglandin to bind to its receptor. Since it is the binding of prostaglandin to its receptor which appears to allow the change in cell-cell adhesion [16], the myoblast membrane order change is likely more directly associated with that event. Although, the myoblasts with added prostaglandin formed gyrotory aggregates by 15 h (not shown), the characteristic 15 h peak of membrane order [15] was not rescued by exogenous prostaglandin. We had previously suggested the this membrane order peak was associated with recovery of the myoblasts from the effects of trypsin and gyrotory

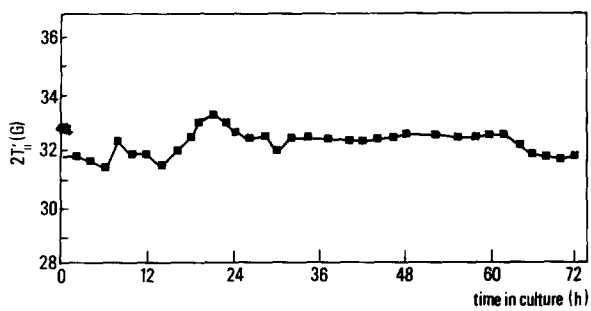

Fig. 3. Myoblast membrane order as represented by $2T_{\parallel}'$ using the 16-nitroxystearate spin-label plotted as a function of time in culture. Each time-point represents the average value of five measurements each of $2T_{\parallel}'$ from five separate experiments. The plot was obtained as in Fig. 1.

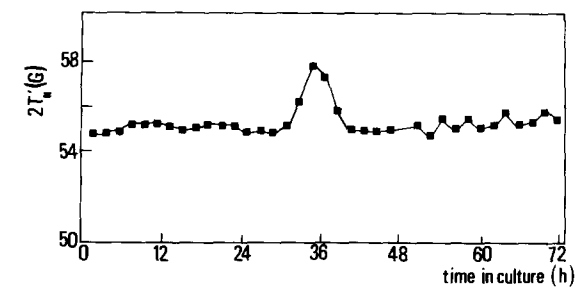

Fig. 5. Myoblast membrane order as represented by $2T_{\parallel}'$ using the 5-nitroxystearate spin-label plotted as a function of time in culture. Both indomethacin (30 μM) and prostaglandin E_1 (1 μM) [16] were added at zero-time in culture. The plot was obtained as described in Fig. 1.

aggregation [15]. However, prostaglandin rescues gyrotory aggregation of the myoblasts but not the 15 h membrane order peak. Thus, our initial attribution cannot be entirely correct. At this time, we can only correlate this initial membrane order change with the synthesis and secretion of prostaglandin by the myoblasts.

The results are consistent with our hypothesis that binding of prostaglandin (possibly prostacyclin) to its receptor is critical for normal myogenesis. They complement our parallel investigations of myoblast membrane electrical properties which suggest that these properties change only after prostaglandin-prostaglandin receptor interaction, possibly at the time of bilayer fusion [17]. Finally, the results raise the possibility that interference with the timely availability of prostaglandins or their receptors may affect early muscle morphogenesis.

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