The Control of Chick Myoblast Fusion by Ion Channels Operated by Prostaglandins and Acetylcholine

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Abstract. Chick myoblast fusion in culture was investigated using prostanoid synthesis inhibitors to delay spontaneous fusion. During this delay myoblast fusion could be induced by prostaglandin E (PGE), by raising extracellular potassium and by addition of carbachol. Carbachol-induced fusion, but not PGE-induced fusion, was prevented by the acetylcholine receptor blocker α-bungarotoxin. Fusion induced by any of these agents was prevented by the Ca channel blockers lanthanum and D600. The threshold for potassium-induced fusion was 7-8 mM; maximal fusion occurred at 16-20 mM. Low extracellular potassium inhibited spontaneous fusion. Intracellular potassium in fusion competent myoblasts was 101 m-moles/l cell. Calcium flux measurements demonstrated that high potassium increased calcium permeability in fusion-competent myoblasts. A 30-s exposure to high potassium or PGE was sufficient to initiate myoblast fusion.

Anion-exchange inhibitors (SITS and DIDS) delayed spontaneous myoblast fusion and blocked fusion induced by PGE, but not carbachol. Blocking the acetylcholine receptor shifted the dose-response relation for PGE-induced fusion to higher concentrations. PGE-induced fusion required chloride ions; carbachol-induced fusion required sodium ions. Provided calcium channels were available, potassium always induced fusion. We conclude that myoblasts possess at least three, independent pathways, each of which can initiate myoblast fusion and that the PGE-activated pathway and the acetylcholine receptor-activated pathway act synergistically.

We suggest that fusion competent myoblasts have a high resting membrane potential and that fusion is controlled by depolarization initiated directly (potassium), by an increase in permeability to chloride ions (PGE), or by activation of the acetylcholine receptor (carbachol); depolarization triggers a rise in calcium permeability. The consequent increase in intracellular calcium initiates myoblast fusion.

The early development of skeletal muscle follows a predictable sequence of events both in vivo and in vitro. Initially the myoblasts divide and align along their long axes. This is followed by an increase in the duration of the myoblast cell cycle in preparation for cytodifferentiation and fusion into myotubes. Myoblast fusion is temporally closely linked to the appearance of muscle specific proteins such as actin, myosin, creatine phosphokinase, and the acetylcholine receptor (for review see Wakelam, 1985), although this link is not obligatory (Adamo et al., 1976). Contractile proteins and action potential generation, and therefore electrically driven contractile activity, also appear shortly after fusion. These early events can take place in the absence of innervation, although the subsequent differentiation of the myofibre is closely dependent on innervating motoneurones.

In the developing embryo there is considerable asynchrony among groups of differentiating myoblasts, which can be avoided by examining these early, innervation independent events in tissue culture. The mechanisms underlying most of these events are unknown.

The link between myoblast fusion and the appearance of muscle specific proteins can be dissociated either by removing calcium ions from the bathing medium (Shainberg et al., 1969) or by inhibiting prostanoid synthesis (Entwistle et al., 1986). In both situations spontaneous myoblast fusion is substantially delayed while the appearance of muscle specific proteins is unaffected. An obligatory role for calcium ions in myoblast fusion is suggested by the requirement for calcium in the culture medium (Shainberg et al., 1969) and evidence showing that entry of calcium into the myoblast may be necessary for fusion to take place (David et al., 1981). The identity of the natural signal which provokes calcium entry has been more elusive, but the specific delay of fusion achieved by inhibiting prostanoid synthesis suggests that prostaglandins could be involved (Entwistle et al., 1986).
The inhibition of myoblast fusion brought about by reducing prostanooid synthesis can be reversed by Prostaglandin E₂ (PGE₂)\(^1\); this offers the opportunity to explore the mechanism activated by PGE₂ in more detail. Our attention has focussed on the possibility that the opening of ion channels in the myoblast membrane might control calcium entry and therefore myoblast fusion. Preliminary reports of some of these results have been presented to The Physiological Society (Entwistle et al., 1983; Bevan et al., 1985).

Materials and Methods

Myoblast Cultures

Myoblasts were obtained by dissection of the developing muscle mass from the hind limbs of 12 d chick embryos. After dissection the tissue was minced with fine scissors, dispersed by mechanical agitation, filtered twice through nylon gauze and then plated in 35-mm collagen-coated petri dishes. Contamination by fibroblasts was below 20%. The cells were plated at a density of 1.4 \(\times\) 10⁶ cells per culture dish in Medium 199 supplemented with 10% horse serum and 2% embryo extract at pH 7.2-7.4 and cultured in a 5% CO₂, >95% humidity atmosphere at 37°C. Embryo extract was prepared by homogenizing 12 d chick embryos and the homogenate diluted 1:1 with Earle's salt solution.

Culture Assay

 Cultures were normally maintained for 54 h. Cell growth and alignment were routinely determined from 24 h by counting 16 fields of live cultures under phase contrast to ensure that additions of drugs or manipulation of the ionic content of the medium did not affect alignment or lead to poor growth or cell death. Cells were considered to be aligned if they were bipolar, had their longitudinal axes parallel with at least two other cells and had contact with one of these cells. Alignment first became substantial at 20-30 h and >80% of the cells were aligned by 44-48 h, when substantial fusion began. Once fusion (assessed retrospectively, see below) had reached 10% measurements of alignment were discounted. In cultures assessed as highly aligned, but not yet fused, random measurements of input capacitance gave values appropriate for single cells; the input capacitance became large once fusion had occurred (Ogden, D., unpublished results). In general 40-50% of the myoblasts had fused at 54 h. When cultures were maintained for longer times, 70-80% fusion had occurred by 70-80 h.

 Cultures were fixed for estimation of fusion at the appropriate time point with glutaraldehyde and stained with Giemsa. Shrinkage during fixation caused individual cells to pull away from each other, assisting the identification of fused, multinucleate cells. Comparison of cell number before or after fixation showed that cell loss during fixation and staining was negligible. Counts were made of the total number of nuclei (\(N_\text{t}\)) and the number of nuclei in multinucleate cells (\(N_\text{n}\)) to allow calculation of the proportion of cells that had fused (\(N_\text{f}/N_\text{t}\)) on the basis of more than 500 cells in 8 microscope fields for each culture dish. Each value reflects the mean ± SD of four identical culture dishes.

For comparison between experiments it was sometimes convenient to calculate a fusion index: % experimental fusion/% spontaneous fusion in controls.

1. Abbreviation used in this paper: PGE₂, prostaglandin E₂.

| Table I. Ionic Composition of Defined Media Used for Ionic Substitution Experiments |
|----------------------------------|---|---|---|---|---|
| **Solution** | **Composition** | **Na** | **K** | **N-methyl-d-glucamine** | **Ca** | **Cl** | **Glucuronate** |
| 1 | Normal | 156 | 4 | -- | 2 | 150 | -- |
| 2 | Low sodium | 1 | 4 | 155 | 2 | 150 | -- |
| 3 | Low Na, low Cl | 1 | 4 | 155 | 2 | 1 | 149 |
| 4 | Low Na, low Cl, low Ca | 1 | 4 | 155 | 0.24 | 1 | 149 |
| 5 | Low Na, low Cl, high K | 1 | 159 | -- | 0.24 | 1 | 149 |
| 6 | High K, low Cl | 1 | 159 | -- | 0.24 | 1 | 149 |

All solutions also contained 2% Dextran (average molecular weight 81,500 D; Sigma), Eagles' complete amino acids, Eagles' complete vitamins (Gibco, Paisley), 5 mM Hepes and the pH was adjusted to 7.3-7.4. Bicarbonate was reduced to 1/10 of the normal level in Eagles' medium and cultures were maintained in air.

Drug Addition and Ionic Manipulations

A complete medium change was carried out routinely after 24 h of culture. All reagents were made up immediately before use. To inhibit prostanooid synthesis cultures were dosed at 30 h with either the cyclo-oxygenase inhibitor Indomethacin (2 \(\times\) 10⁻⁶ M) or the phospholipase A₂ inhibitor Chloroquine (5.6 \(\times\) 10⁻³ M). A second dose was added at 44 h (1 \(\times\) 10⁻⁴ M Indomethacin or 1.8 \(\times\) 10⁻⁴ M Chloroquine). All other drugs were added to the culture dishes at 24 or 44 h of culture unless otherwise indicated. Controls received an equivalent volume of the solution in which the drugs were suspended. The following reagents were used: Prostaglandin E₂ (Sigma Chemical Co. London, U.K.), D600 (\(\alpha\)-isopropyl-\(\alpha\)-(N-methyl-N-homoveratryl)-\(\alpha\)-aminopropyl)-3,4,5-trimethoxyphenylacetonitrite hydrochloride, gift of the late P.F. Baker), carbachol (Sigma Chemical Co.), \(\alpha\)-bungarotoxin (Boehringer, U.K.) SITS (Sigma Chemical Co.) and DIDS (Sigma Chemical Co.) at the concentrations indicated in the Results section. All inorganic salts came from B.D.H. U.K. Ltd. Lanthanum was added as the chloride.

When potassium in the culture medium was raised at 24 h either K replaced sodium mole for mole or excess potassium was added as potassium glucuronate to avoid alterations in the chloride equilibrium potential. When potassium was increased close to (44 h in culture) or during spontaneous fusion (52 or 54 h in culture) it was added as excess KCl. When required sodium ions were replaced by N-methyl-D-glucamine and chloride ions by glucuronate. Low calcium solutions contained ~0.24 mM calcium. The composition of the defined media used for manipulations of ionic composition are given in Table 1.

The concentration of potassium in normal medium and in media where potassium levels had been altered was determined by flame photometry. Normal medium contained 6-6.5 mM potassium after addition of horse serum and embryo extract.

Alterations in the ionic composition of the medium normally were done at 24 h to avoid induction of myoblast fusion (see Results). When it was desirable to alter the composition of the medium at times very close to or during spontaneous fusion the medium change was made in low calcium solution and Ca added back just before the addition of drugs or potassium.

Cell growth and alignment were unaffected by all drugs and ionic manipulations.

Measurements of Calcium Efflux

\(^4\)CaCl₂ (20 \(\mu\)Ci/dish containing 1.2 ml) was added to the culture dishes at 24 h. SITS and \(\alpha\)-bungarotoxin were added at 40 h to inhibit spontaneous myoblast fusion. At 54 h, when spontaneous fusion in uninhibited, parallel cultures was approaching 45%, the radiolabeled medium was removed and each dish was rapidly rinsed 5 times with 1 ml of inactive solution of the same composition as the load solution to remove the bulk of extracellular labelled calcium and the collection of efflux data then began. The bathing solution (1 ml) was rapidly withdrawn and replaced with an equivalent volume at intervals of 2 min. After the resting efflux of calcium had been established (10 min, 5 samples) the potassium concentration of the wash solution was increased to 24 mM and two efflux samples taken. The wash solution was then returned to normal potassium (6 mM) for a further two samples. At the end of the efflux period the cells were solubilized in 1 ml of wash solution containing 0.1% Triton X-100 to allow the amount of radioactive calcium remaining in the cells to be determined. The samples were taken up into 3 ml of Ready-solv (Beckman, High Wycombe, U.K.) and counted in a liquid scintillation counter. The rate constant for each period of efflux (\(k\)) was calculated from the ratio of activity lost per minute to the arithmetic mean radioactive content of the cells (determined from the radioactivity re-
had been treated with the prostanoid synthesis inhibitors chloroquine (●) or indomethacin (▲) are also included. Each point gives the mean of 4 parallel culture dishes; the bars indicate ±1 SD.

**Determination of Intracellular Potassium**

Cultures were taken shortly before the beginning of spontaneous fusion. The culture medium was removed and the cells rinsed rapidly 5 times with potassium-free, 150 mM NaCl, 2 mM CaCl₂, 2 mM MgCl₂, solution buffered with 2.5 mM Hepes to pH 7.4. After the final rinse, as much solution as possible was removed and replaced by 0.5 ml of 0.1% Triton X-100 to lyse the cells. After 20 min this solution was removed and replaced by a second aliquot of 0.5 ml of 0.1% Triton X-100 to ensure complete solubilization. The aliquots were pooled and analysed for potassium in an atomic absorption spectrophotometer (Perkin Elmer, model No. 2380). The concentration of potassium in the final rinse solution was negligible, indicating that all extracellular K had been removed. The efficiency of the rinsing procedure was further tested by exposing the cells to 12 mM K medium for 30 min and then carrying out the rinsing procedure; the K content of the final rinse was again negligible.

Cell number for each culture dish was determined by counting nuclei under phase contrast in 16 fields (0.6% of the total area) in each of 8 dishes (~1000 cells/dish). An average cell volume was calculated from the length (x,1000 cells/dish). An average cell volume was calculated from the length and maximum diameter of 100 cells selected at random from each of 6 dishes, considering each cell to be a cylinder, which overestimates the true cell volume. Potassium was assumed to remain in the cells at the end of the efflux period and the appropriate cumulative loss of radioactivity.

**Results**

Fig. 1 also shows measurements from cultures which had taken place in controls. The features of the inhibition imposed on fusion by prostanoid synthesis inhibitors are considered in detail elsewhere (Entwistle et al., 1986). Comparisons between cultures were therefore made at 54 h of culture, when 40-50% fusion had taken place in controls. The features of the inhibition imposed on fusion by prostanoid synthesis inhibitors are considered in detail elsewhere (Entwistle et al., 1986).

**The Role of Calcium Entry**

The calcium channel blockers D600 and lanthanum prevented the fusion-inducing action of PGE₁ in cultures treated with prostanoid synthesis inhibitors (Table I), suggesting that calcium entry is involved. Both reagents also inhibited spontaneous fusion (see Entwistle et al., 1986). The effective range for D600 fits those normally used to block calcium channels (e.g., Callan and Keenan, 1984; Turner and Golling, 1985). The concentrations of lanthanum are higher than those normally used to block calcium channels (10⁻⁵ - 10⁻⁴ M). However the free ion concentration of lanthanum must have been well below the added level because precipitation of lanthanum by other constituents of the medium always occurred. The reduction of spontaneous fusion by either Ca channel blocker could be overcome by raising the calcium concentration to 5.6 mM.

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**Table II. D600 and Lanthanum Prevent Myoblast Fusion**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Fusion mean ±SD</th>
<th>No. of experiments</th>
<th>No. of dishes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>47.4 ± 4.0</td>
<td>11</td>
<td>44</td>
</tr>
<tr>
<td>D600 2.0 mM</td>
<td>17.3 ± 4.9</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>0.2 mM</td>
<td>20.0 ± 1.2</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>0.06 mM</td>
<td>45.1 ± 1.0</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Lanthanum 1.0 mM</td>
<td>10.8 ± 3.6</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>0.3 mM</td>
<td>21.6 ± 4.4</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>0.1 mM</td>
<td>42.2 ± 2.0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>15.1 ± 4.5</td>
<td>6</td>
<td>24</td>
</tr>
<tr>
<td>Chloroquine + PGE₁</td>
<td>51.5 ± 3.0</td>
<td>6</td>
<td>24</td>
</tr>
<tr>
<td>Chloroquine + D600</td>
<td>15.7 ± 2.2</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Chloroquine + Lanthanum + PGE₁</td>
<td>9.8 ± 4.3</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>18.6 ± 3.5</td>
<td>7</td>
<td>28</td>
</tr>
<tr>
<td>Indomethacin + PGE₁</td>
<td>52.6 ± 3.3</td>
<td>7</td>
<td>28</td>
</tr>
<tr>
<td>Indomethacin + D600</td>
<td>13.4 ± 3.7</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Indomethacin + Lanthanum + PGE₁</td>
<td>15.3 ± 3.9</td>
<td>2</td>
<td>8</td>
</tr>
</tbody>
</table>

All estimates of myoblast fusion made at 54 h of culture. Chloroquine and indomethacin: concentrations and dosing schedule as in the Materials and Methods section; PGE: 10⁻⁹ M, D600: 2.0 mM (except as otherwise indicated); Lanthanum: 1 mM (except as indicated).
gives the mean + 1 SD for 4 dishes. Arrow indicates the likely concentration of potassium in the experiments of Okazaki and Holtzer.

The relation between extracellular potassium concentration and fusion for two experiments on chloroquine-inhibited cultures. The mean level of spontaneous fusion in normal medium (6 mM potassium) was set at one and the remainder of the results expressed as a fusion index (% experimental fusion/% spontaneous fusion). So long as extracellular potassium remains at or below 5.6 mM the inhibition is maintained. At 8 mM K, fusion reached 60% of the control. 16 mM potassium was sufficient to override completely the inhibitions and brought fusion to levels slightly above spontaneous fusion. Thus the threshold for potassium-induced fusion must lie close to 7 mM. Almost identical dose-response relations were obtained in 2 experiments with indomethacin inhibited cultures. The sensitivity of myoblast fusion to relatively small increases in extracellular potassium suggests that potassium induces fusion through an effect on the membrane potential, which should be highly sensitive to potassium concentration in this range.

The role of the membrane potential

Calcium entry into the cell can be controlled by the membrane potential or through some other mechanism. Membrane depolarization can be achieved by raising extracellular potassium, the magnitude of the depolarization being related
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or prostaglandin to initiate fusion, made on indomethacinoccupied by water, then \( [K] \) would be 144 mmol/kgm.

The Speed of Action of Potassium and PGE

Fig. 3 shows experiments to determine how long fusion-inhibited myoblasts have to be exposed to either potassium or prostaglandin to initiate fusion, made on indomethacin- or chloroquine-inhibited cultures at 54 h. The effective period of exposure to either agent was terminated by the addition of lanthanum to prevent further entry of calcium. The cultures were maintained for a further two hours before fixation and calculation of the proportion of cells that had fused.

When lanthanum was added 30 s before potassium (a) or PGE, (b) the block of myoblast fusion was maintained. However, both for potassium and PGE, a 30-s exposure was sufficient to initiate fusion close to the level observed in uninhibited, parallel cultures. Addition of lanthanum to-

Figure 3. Potassium and Prostaglandin E\(_1\) induce myoblast fusion rapidly. (a) The speed of action of potassium. Ordinate: fusion index. Abscissa: Time after addition of potassium. (b) The speed of action of PGE\(_1\). Ordinate: Myoblast fusion. Abscissa: Time after addition of PGE\(_1\). In both parts of the figure the action of the fusion-inducing agent was terminated by the addition of lanthanum. (a) chloroquine inhibited cultures (c) indomethacin inhibited cultures. Points to left of zero time give fusion level at 54 h of culture in parallel prostanoid synthesis-inhibited cultures. (Open and solid circles) Spontaneous fusion in each experiment. Lines drawn through points by eye. Each point gives the mean ±1 SD of four culture dishes.

The acetylcholine receptor agonist, carbachol, used in fusion with K or PGE\(_1\)-induced variable degrees of fusion, reflected in the large standard deviation, probably because of differing speeds of diffusion. The speed of action of both potassium and PGE is therefore very fast, and within that to be expected on the basis of diffusion within the culture dish. Such results suggest that both fusion-inducing agents act almost instantaneously, consistent with their effects operating through membrane depolarization.

Activation of the Acetylcholine Receptor also Initiates Myoblast Fusion

If the membrane potential is a major factor controlling myoblast fusion then any agent which depolarizes the myoblast should reverse inhibitions of fusion imposed by prostanoid synthesis inhibitors. Myoblasts possess functional acetylcholine receptors, which specifically bind \( \alpha \)-bungarotoxin, before fusion begins (Entwistle et al., 1988). Fig. 4 a shows that the acetylcholine receptor agonist, carbachol, used in preference to acetylcholine because it is not hydrolyzed (see for example Gardner et al., 1984), induced myoblast fusion in prostanoid synthesis-inhibited cultures. This induction was blocked by Ca channel inhibitors (Fig. 4 a). \( \alpha \)-bungarotoxin, a specific blocker of acetylcholine receptors (Chang and Lee, 1963), prevented the fusion inducing action of carbachol, confirming that carbachol acts through the acetylcholine receptor. An example is included in Fig. 4 a, which shows also that potassium remained able to initiate fusion in the presence of \( \alpha \)-bungarotoxin. The last part of Fig. 4 a shows that PGE\(_1\) remained effective despite the presence of \( \alpha \)-bungarotoxin.
The ability of prostanoid synthesis inhibitors to delay spontaneous myoblast fusion has led to the suggestion that a prostanoid might be a natural fusion inducing agent (Entwistle et al., 1986). Spontaneous myoblast fusion may involve the acetylcholine receptor also since either α-bungarotoxin or an alternative acetylcholine receptor blocker, Naja naja toxin, delayed fusion in control cultures (see accompanying paper: Entwistle et al., 1987b). Fig. 5 compares the dose–response relation for Prostaglandin E₁-induced fusion in prostanoid synthesis-inhibited cultures in the presence and absence of functional acetylcholine receptors. The dose–response relation for PGE₁ in the presence of prostanoid synthesis inhibitors alone is taken from results of Entwistle et al. (1986). The midpoint lies at $3.2 \times 10^{-6}$ M PGE₁ and maximum fusion is attained at $5.6 \times 10^{-8}$ M. In the presence of α-bungarotoxin the dose–response relation for PGE₁ shifts to higher concentrations of PGE₁ in the presence of α-bungarotoxin. Note shift to higher concentrations of PGE₁ in the presence of α-bungarotoxin.

The Ionic Basis of ACh and PGE₁-induced Myoblast Fusion

Activation of the acetylcholine receptor increases membrane permeability to sodium and potassium ions, with membrane depolarization depending on the relative increase in permeability to Na; carbachol is known to act similarly (for example see Gardner et al., 1984). Fig. 4 b shows that removing Na ions from the culture medium prevents carbachol-induced fusion. Extracellular sodium in the culture medium was replaced with N-methyl-D-glucamine, a large, membrane impermeant cation (Bevan et al., 1984). Potassium-induced fusion was unaffected. This experiment shows also that PGE₁-induced fusion was unaffected by Na removal, reinforcing the suggestion that carbachol and prostaglandins act through separate pathways.

In all experiments described thus far, any change in the medium on indomethacin- or chloroquine-inhibited cultures was carried out before spontaneous fusion was apparent in control cultures because at later times the medium change itself induced fusion, despite the continued presence of prostanoid synthesis inhibitors. During experiments in which Na was replaced by an impermeant cation we found that changing a sodium-free medium did not induce fusion. Fig. 6 plots fusion induced by a medium change at 52 h against the concentration of sodium in the medium. The cells were fixed, and the level of fusion assessed, 2 h later. The indomethacin or chloroquine inhibition was maintained when extracellular sodium was 18 mM or less and released completely if sodium was at or above 30 mM, as if the medium change caused an increase in Na permeability. The fall in external sodium presumably reduced inward current so that eventually there was no longer sufficient depolarization to initiate myoblast fusion. The effect was abolished also by α-bungarotoxin, suggesting that the medium change somehow activated the acetylcholine receptor.

Prostaglandin E₁ does not require external sodium ions to induce fusion (Fig. 4 b). To explore the possible involvement of chloride ions in PGE₁-induced fusion we tested the effect of SITS and DIDS, which inhibit Cl/HCO₃ exchange (Knauf and Rothstein, 1971) and block chloride channels (Gray and Ritchie, 1986). Fig. 7 shows that either SITS or DIDS prevented induction of fusion by the prostaglandin. However, both carbachol and potassium remained able to induce fusion showing that the inhibitors had not interfered with the state of the cells. Both SITS and DIDS inhibited also spontaneous myoblast fusion: cell number and cell alignment were not affected. As with prostanoid synthesis inhibitors (Entwistle et al., 1986) and α-bungarotoxin (Entwistle et al., 1988), the inhibition was not permanent and spontaneous fusion commenced after a delay of ~12 h. SITS produced 50% inhibition at $5 \times 10^{-4}$ M, and maximal inhibition at $10^{-3}$ M while DIDS gave 50% inhibition at $7 \times 10^{-3}$ M with the maximum at $5 \times 10^{-3}$ M. These concentrations are close to those used either to block Cl/HCO₃ exchange or chloride channels.

Fig. 7 b shows that when chloride ions were replaced by
Figure 7. Anion exchange inhibitors and chloride channel blockers prevent myoblast fusion. (a) SITS and DIDS inhibit PGE₁-induced fusion in cultures inhibited with prostanoid synthesis inhibitors. Ordinate: fusion index. Results from two separate experiments combined. Bars give: spontaneous fusion; fusion in SITS alone; PGE₁-induced fusion in the presence of SITS; potassium-induced fusion in the presence of SITS; carbachol-induced fusion in the presence of SITS; fusion in DIDS alone; PGE₁ in the presence of DIDS; potassium-induced fusion in the presence of DIDS; carbachol-induced fusion in the presence of DIDS; Spontaneous fusion given as mean ±1 SD of eight dishes; other conditions mean ±1 SD of four dishes. (b) Prostaglandin E₁-induced fusion requires the presence of chloride ions. Ordinate: myoblast fusion. The first bar gives the level of spontaneous fusion in untreated cultures, otherwise the results came from indomethacin-inhibited cultures. For the next three panels, the calcium concentration in the culture medium was reduced to 0.24 mM during the change to sodium-free and chloride-free (N-methyl-D-glucamine gluconate) medium to prevent induction of fusion. Calcium was restored to 2 mM just before the addition of PGE₁ or 22 mM potassium. (Second panel) Fusion in indomethacin alone. (Third panel) PGE does not induce fusion. (Fourth panel) Fusion induced by 22 mM potassium. (Final panel) changing from chloride-containing to chloride-free medium, in the presence of 2 mM calcium, induces myoblast fusion.

the relatively impermeant gluconate anion (Woodbury and Miles, 1973), with appropriate precautions to avoid the induction of fusion by any change in medium (see legend to Fig. 6). PGE₁ was no longer able to induce myoblast fusion in cultures treated with prostanoid synthesis inhibitors. 22 mM potassium nevertheless restored fusion to spontaneous levels. The final bar shows that, in the absence of sodium, a medium change from chloride-containing to chloride-free solution will induce myoblast fusion.

**Potassium Induces an Increase in Calcium Permeability of the Myoblast Membrane**

Myoblast fusion induced by raising extracellular potassium could be prevented by the Ca channel blockers D600 and lanthanum, suggesting that potassium provokes an increase in calcium permeability, leading to calcium entry into the myoblast. This was tested by monitoring the efflux of ⁴⁰Ca from myoblasts at normal and increased levels of extracellular potassium. The acetylcholine receptor was blocked by α-bungarotoxin and the prostanoid-activated pathway inhibited with SITS. Under these conditions spontaneous fusion is inhibited, potassium-induced fusion is maximal and a medium change has no effect. The myoblasts were loaded with ⁴⁰Ca to equilibrium by adding radiolabeled calcium at 24 h of culture, so that the efflux rate reflects the calcium permeability.

Figure 8 plots the rate constant for calcium efflux into inactive wash solution of the same ionic composition as the loading solution. Efflux measurements began at 54 h of culture, when spontaneous fusion in parallel cultures had reached the usual 40–50%. The washout of labelled calcium from the extracellular space was complete after 2 min and over the next 8 min calcium left the cells with an average rate constant of 0.12 min⁻¹. At the bar the potassium concentration in the wash solution was increased to 24 mM for 4 min (2 samples). This produced a prompt fourfold increase in the efflux of Ca, indicating a substantial rise in calcium permeability. The efflux rate began to fall away during the second sample period, suggesting that the permeability to calcium may inactivate even during short exposures to potassium, as found in similar experiments on the adrenal medulla (Baker and Rink, 1975). On return to wash solution with 6 mM potassium the Ca efflux returned to its initial level. Similar increases in Ca efflux on raising extracellular K to 24 mM were observed in 4 other experiments.

**Discussion**

Pre-fusion myoblasts contain at least two pathways that can initiate the cascade of events leading to myoblast fusion. One operates through the acetylcholine receptor, the other through a mechanism activated by the prostanoid Prostaglandin E₁. Fusion initiated by the acetylcholine receptor requires extracellular sodium ions and fusion initiated by the prostanoid requires chloride ions. An increase in extracellular potassium can also initiate myoblast fusion. All three fusion-inducing agents depend on the presence of functional calcium channels in the myoblast membrane. The simplest hypothesis to explain these findings is that in each case
the first step causes membrane depolarization which, in turn, leads to calcium entry.

A Role for Calcium Ions

A role for calcium ions in normal fusion was previously indicated by the inhibitory effect of removing calcium from the culture medium (Shainberg et al., 1969) and David et al.'s (1981) finding that the calcium channel blocker D600 inhibited spontaneous fusion, while the calcium ionophore A23187 induced precocious fusion. Furthermore David et al. (1981) found an increase in calcium influx both during spontaneous myoblast fusion and during precocious fusion initiated by either A23187 or relatively high (10^{-4} M) concentrations of PGE_{1} (David and Higginbotham, 1981). The present results strengthen the case for the intimate involvement of calcium ions in myoblast fusion. Thus fusion in prostanoid synthesis inhibited cultures initiated by low concentrations of Prostaglandin E_{1} (10^{-4} M), carbachol or potassium can be prevented by calcium channel blockers. An increase in extracellular potassium induced a substantial increase in calcium efflux from fusion competent, but inhibited, myoblasts, providing direct evidence for an increase in calcium permeability. In the adrenal medulla, the increase in calcium permeability elicited by potassium-induced depolarization is transient (Baker and Rink, 1975). Similar inactivation of the Ca permeability occurred in myoblasts. However such inactivation is probably irrelevant for the control of fusion, since exposure to potassium for 30 s was sufficient to evoke a full response and the response, once triggered, may be a single event in the life history of each fusion competent myoblast.

A Role for Membrane for Depolarization

A role for membrane depolarization in myoblast fusion is indicated by the finding that both fusion arrested myoblasts and normal myoblasts are exquisitely sensitive to the extracellular concentration of potassium ions. Fusion was initiated at \( \sim 7 \) mM potassium, and restored to control levels at 16 mM potassium. Raising potassium did not provoke substantial precocious fusion, which implies that either the insertion into the membrane of any voltage-dependent element of the fusion process or the acquisition of a relatively high membrane permeability to potassium occurs shortly before fusion is initiated. Our results differ from those of Schudt et al. (1973), who raised extracellular potassium from 24 h in culture by adding 80 mM KCl to the medium and found spontaneous myoblast fusion to be inhibited by \( \sim 30\% \). In the long term adding excess KCl will not only alter the potassium equilibrium potential, but also the chloride equilibrium potential. The results of Schudt et al. (1973) may have arisen from changes in chloride (see below) rather than potassium distribution; secondary effects may have intervened also.

Mechanisms that are highly sensitive to potassium, and operate through membrane depolarization and calcium entry, have previously been reported (e.g., Ginesi et al., 1983). Measurement of intracellular potassium showed that fusion-competent myoblasts contain 100-140 mM potassium, which gives a potassium equilibrium potential of \(-80 \) to \(-89 \) mV. If the membrane selected for potassium to the same degree as in adult muscle, moving from 5.6 to 16 mM potassium would produce about a 25 mV depolarization, which should be sufficient to open voltage-dependent membrane channels. For example, the low voltage activated calcium channel, which has been found in a number of cell types including chick muscle, is activated \(+15 \) mV depolarized to a resting potential of \(-75 \) mV (Miller, 1986). However a membrane potential change of this magnitude implies that the resting potential of the immediately pre-fusion myoblast is high, in the range of \(-70 \) to \(-80 \) mV at 5.6 potassium. Most published reports suggest a membrane potential of between \(-12 \) and \(-60 \) mV in myoblasts, which would not be adequate to account for the observed potassium sensitivity. However Kidokoro (1975) concluded that the resting potential of a clonal myoblast cell line (L6) was close to \(-74 \) mV, with the potassium equilibrium potential \(-83 \) mV at 5.6 mM K.

If depolarization is important then as the membrane potential becomes more negative it should be progressively more difficult to initiate fusion. Spontaneous myoblast fusion was progressively reduced at extracellular potassium concentrations below threshold, in a manner described by the Goldman equation using assumptions that set the membrane potential in 5.6 mM potassium at \(-72 \) mV. The substantial potassium sensitivity suggests that intracellular sodium is \( \sim 20 \) mM. A low intracellular sodium concentration is strongly correlated with high intracellular levels of potassium, a relatively potassium-permeable membrane and a high resting potential (Williams, 1970). Potassium sensitivity is probably acquired close to the time of initiation of spontaneous fusion. A high resting potential in immediately pre-fusion myoblasts therefore could easily have been missed in previous studies since there is no way of identifying morphologically an aligned myoblast that is within minutes of fusion from one which is several hours away.

The overall conclusion is that the resting membrane potential of the immediately prefusion myoblast is probably high, with a substantial membrane permeability to potassium relative to that to other ions, and that an increase in extracellular potassium concentration initiates myoblast fusion by depolarizing the membrane, leading to the opening of voltage-dependent calcium channels. The entry of calcium triggers other events in the myoblast which lead to membrane fusion.

The sensitivity of myoblast fusion to the extracellular potassium concentration focuses attention on the levels of potassium in media used to culture myoblasts. The medium used in the present experiments contained 5.6 or 6 mM potassium; media used by others seem likely to have contained higher concentrations (e.g., 8-10 mM; Okazaki and Holtzer, 1966). Inhibition or delay of myoblast fusion will be virtually impossible in such solutions, except by removing extracellular calcium or blocking calcium channels. Potassium in the intracellular fluid of the fetal limb, which will surround myoblasts during development, is probably in the region of 5-6 mM, as in serum, a level which permits maximum myoblast fusion once the natural fusion-inducing agent is available.

Ionic Mechanisms of Receptor Activation

It is probable that the acetylcholine receptor agonist carbachol initiate fusion by depolarizing the myoblast membrane. Carbachol loses its fusion-inducing ability when sodium ions are omitted from the medium, which is strong evidence that carbachol causes depolarization. The suggestion that carbachol produces depolarization by activating the
Acetylcholine receptor blockers. Thirdly, myoblasts showed a carbachol-induced increase in permeability to guanidinium ions, which are likely to behave like sodium ions. There is extensive evidence to show that one consequence of nicotinic acetylcholine receptor activation is an increase in membrane permeability to sodium ions. While the present results cannot rule out some mechanism that depends on extracellular sodium but does not require depolarization, the experimental findings are entirely consistent with such a hypothesis.

Prostaglandin E₁ requires chloride ions to induce myoblast fusion and reagents known to block chloride channels prevent both the fusion-inducing action of the prostanooid and spontaneous myoblast fusion. The evidence for the direct involvement of chloride ions is, therefore, strong. The likelihood that PGE causes an increase in chloride permeability that leads to membrane depolarization can, for the present, be assessed only by indirect argument. PGE acts as rapidly as potassium, which is consistent with an effect on the membrane potential. Changing from chloride-containing to chloride-free solution initiated myoblast fusion: replacement of chloride with an impermeant anion produces a transient depolarization (see Hodgkin and Horowicz, 1959). An increase in chloride permeability will cause depolarization if intracellular chloride is maintained above the concentration predicted for a passive distribution. Although the situation for chick myoblasts is not known, both frog and mammalian muscle fibres possess an inwardly directed chloride pump which maintains the chloride equilibrium potential depolarized to the resting potential (Bolton and Vaughan-Jones, 1977; Betz et al., 1986). Intracellular chloride in myoblasts would have to be about 25 mM at a resting potential of -70 to -80 mV to achieve a depolarization of 20-30 mV when the chloride permeability becomes dominant. A chloride pump would, therefore, need to maintain Cl⁻ around 15 m-moles away from $E_{Cl}^-$. The chloride permeability of myoblasts has not been measured, but differentiating rat and chick myotubes have a low resting chloride permeability (Kidokoro, 1975; Schwarz and Kolbe, 1984). Chloride accumulation to the degree required therefore seems likely to be within the capacity of the myoblast. Finally, chloride-dependent action potentials have been reported in very young myotubes (Fukada, 1974). Firm evidence that prostaglandin induces fusion by increasing chloride permeability, with accompanying depolarization, must await exploration of the membrane properties of the immediately pre-fusion myoblast.

The suggestion that an increase in permeability to sodium ions can trigger calcium entry and consequent myoblast fusion appears to conflict with David et al’s (1981) conclusion that an increase in permeability to small ions other than calcium is not involved in myoblast fusion. This was based on the finding that the sodium ionophore Gramicidin S was unable to promote precocious fusion. This result can be reconciled with ours simply by assuming that the mechanisms controlling voltage-dependent calcium entry are only inserted into the myoblast membrane shortly before spontaneous fusion begins. Gramicidin S would not then be expected to provoke precocious fusion. If this assumption is correct then David et al’s (1981) finding that an increase in Ca brought about by the Ca ionophore A23187 can promote precocious fusion allows the further conclusion that myoblasts are able to respond to a rise in Ca, before the membrane receptors that normally dictate the time course of myoblast fusion are inserted.

It has recently been shown that myoblasts possess membrane receptors for ATP (Kolb and Wakelam, 1983) and we have recently obtained evidence to suggest that they are involved in ensuring successful fusion (Entwistle and Bevan, manuscript in preparation). It is likely that this mechanism also operates through membrane depolarization, as suggested by the recent demonstration of an excitatory action of ATP on chick myoblasts and myotubes (Hume and Honig, 1986).

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