Myoblast Fusion Is Regulated by a Prostanoid of the One Series Independently of a Rise in Cyclic AMP

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Abstract. The role of prostanoids in the regulation of chick myoblast differentiation has been investigated. At 3×10^{-6} M, indomethacin and chloroquine specifically inhibit cell fusion. They do not affect cell proliferation, alignment, or the expression of two muscle-specific proteins, namely, the acetylcholine receptor and the muscle-specific form of creatine phosphokinase. The results demonstrate that it is indomethacin's activity as an inhibitor of prostaglandin synthesis at the cyclyooxygenase step that causes the block of cell fusion, whereas chloroquine probably acts at the earlier step of phospholipase A. Prostaglandin E_1 (PGE$_1$), but not prostaglandin E_2 (PGE$_2$), rapidly reverses the inhibition of fusion imposed by indomethacin or chloroquine. The dose response of the myoblasts to PGE$_1$ is a bell-shaped curve with a 100% reversal of fusion at $\sim 10^{-9}$ M. Eicosatrienoate and linoleate reverse the inhibition of fusion with similar kinetics, whereas arachidonate is completely ineffective. The ability of PGE$_1$ and eicosatrienoate but not PGE$_2$ and arachidonate to restore fusion to control levels implies that fusion is specifically regulated by a prostanoid of the one series. The reversal of the fusion-block by linoleate further suggests that this fatty acid provides the necessary source of eicosatrienoate in the myoblast plasma membrane. At 10^{-8} M and above, PGE$_1$ and PGE$_2$ stimulate adenylate cyclase and depress control fusion as does 10^{-5} M isoproterenol. The β-adrenergic blocker propranolol abolishes both isoproterenol's inhibition of myoblast fusion and its activation of adenylate cyclase. The similar depressions imposed on cell fusion by 10^{-8}–10^{-6} M prostanoid and 10^{-5} M isoproterenol suggest that in both cases the depressive effects are mediated by cyclic AMP. It is concluded that a prostanoid of the one series regulates fusion by a cyclic AMP–independent mechanism.

THE multinucleated skeletal muscle fiber is formed by the fusion of mononucleated precursor myoblasts. Both in vivo and in culture, the bipolar myoblasts initially undergo a phase of proliferation while aligning along their longitudinal axes. Membrane fusion subsequently takes place between the aligned cells. At approximately the same time as the onset of cell–cell fusion, the embryonic muscle cell ceases DNA synthesis and cell division and initiates the elaboration of the specialized proteins associated with skeletal muscle contraction. The relationship between these three events is not yet fully elucidated. However, it is accepted that the expression of muscle-specific proteins can be uncoupled from the fusion event and that once inside a multinucleate cell, the myogenic nuclei are incapable of either DNA synthesis or cell division.

Numerous agents have been identified that inhibit cell–cell fusion (41). Most of these inhibitions probably represent a more general block of differentiation or cellular function. An exception to this is the inhibition of cell fusion achieved by lowering external calcium. Unfortunately, reducing external Ca^{++} to investigate the mechanism of myoblast fusion is of limited use. First, the effects of this manipulation are not always limited to myoblast fusion. The expression of muscle-specific proteins can also be inhibited (5, 30, 39). Second,

inhibiting fusion by limiting the availability of calcium is not an appropriate manipulation for investigation of events that occur before the calcium-dependent step. These two considerations led us to study other methods of achieving specific and reversible inhibitions of the fusion event itself.

Prostanoids (products of the cyclooxygenase pathway including thromboxanes, the prostaglandins, and their metabolites) are implicated in the regulation of myoblast differentiation. Low concentrations of putative inhibitors of prostanoid synthesis prevent the onset of the fusion process (44) and this is reversible by exogenously added prostaglandin E$_1$, (PGE$_1$)[1] (11, 44). However, neither of these inhibitor studies examined the effects of manipulating prostanoid levels on other aspects of differentiation and so the degree of specificity of the effects of PGE on cell fusion is therefore not known.

In this study, we present the results of an investigation into the nature of the inhibitions imposed upon myoblast fusion by low concentrations of putative inhibitors of prostanoid synthesis. Specifically, the effects of indomethacin (an inhibitor of cyclooxygenase) and chloroquine (an inhibitor of

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1. *Abbreviations used in this paper:* PGE$_1$ and PGE$_2$, prostaglandins E$_1$ and E$_2$, respectively.

phospholipase A₂) on muscle-cell fusion, alignment, proliferation, and the expression of acetylcholine receptors and muscle-specific creatine phosphokinase have been examined. Data are presented that assess prostanoid synthesis as the critical site of action of the drugs in their block of myoblast fusion. This mechanism of blocking myoblast fusion is then employed to investigate the capacity of endogenously produced prostanoid to trigger cell fusion. Finally, the role of cyclic AMP in the regulation of myoblast fusion is reexamined.

Materials and Methods

Cell Cultures

Chick myoblast cultures were prepared from Stage 39 chicks (18). The legs were dissected free of skin, bones, and other nonmuscle tissue. The harvested tissue was then finely scissored in complete medium and agitated by vortex mixing (37). A suspension of single cells was prepared by filtering the resultant tissue preparation through two layers of nylon mesh (pore size 30 μm) and repeatedly pipetted immediately before use. Cells were plated out in 1.2 ml of medium at a density of 1.4 × 10⁵ cells per 35-mm collagen-coated petri dish (Falcon Labware, Oxnard, CA). The culture medium contained Medium 199 (Gibco, Grand Island, NY) supplemented with 10% horse serum (Flow Laboratories, Flow General Inc., McLean, VA), 2% embryo extract, 100 U/ml of penicillin, and 100 U/ml of streptomycin (Gibco), and was adjusted to a final pH of 7.4. The cells were cultured at 37°C in 5% CO₂ at 100% humidity. A complete medium change was carried out 24 h after the initial plating.

Drug Additions

Chloroquine, mepacrine, and indomethacin (Sigma Chemical Co., St. Louis, MO) were routinely added as 30 μl of concentrate made up in Earle's salts (Gibco), at ~12-h intervals, commencing after the medium change. Controls were dosed with 30 μl of Earle's salts at each addition. The drugs were made up immediately before use. Linoleic acid (9,12 octadecadienoic acid; Sigma Chemical Co.), eicosatrienoic acid (8,11,14 eicosatrienoic acid; Sigma Chemical Co.), and arachidonic acid (5,8,11,14 eicosatetraenoic acid; Koch-Light Laboratories, Colnbrook, Buckinghamshire, England) were stored as 10⁻² M ethanolic stock solutions at −40°C. Immediately before use, the stock solutions were titrated with ethanolic sodium hydroxide and diluted with acidic Earle's salts (pH 4). 30 μl of the appropriate concentrate was added to the dishes at 48 h in culture. PGE₁ and PGE₂ (Sigma Chemical Co.) were stored as 10⁻³ M ethanolic stock solutions. Immediately before use, these were diluted with Earle's salts and administered to the dishes in a similar manner to the essential fatty acid esters. Isoproterenol, propranolol, and dibutyryl cyclic AMP (Sigma Chemical Co.) were prepared in Earle's salts on the day of use. Isoproterenol and dibutyryl cyclic AMP were added at 48 h of culture and, when appropriate, propranolol was added 30 min before this.

Cell Counts

All counts were performed on an Olympus inverted microscope at a magnification of 200. Cell numbers were estimated by direct microscopic examination of either living cultures under phase-contrast optics or fixed and stained cultures (see below) with bright field optics. In prefusion cultures, the numbers of cells counted by the two techniques were not significantly different (data not presented).

A cell was defined as aligned if it had a bipolar morphology, its longitudinal axis parallel to the longitudinal axes of at least two neighboring cells, and was in contact with at least one of them. Dividing cells were included in the tally of unaligned cells and the counts were performed using phase-contrast optics.

Cell fusion was estimated by microscopic examination of glutaraldehyde-fixed and Giemsa-stained cultures, employing bright field optics. Percentage fusion was calculated by counting the number of nuclei in multinucleated cells, dividing this by the total number of nuclei observed, and multiplying by 100.

To facilitate the comparison of results obtained in separate experiments, results were routinely plotted as a fusion index. The fusion index is the percentage fusion in the treated cultures divided by the percentage fusion in control cultures. All values presented represent the mean ± SD. Where no SD is apparent, it is contained within the point itself.

Adenosine 3′:5′-Cyclic Monophosphate Assay

Cyclic AMP was assayed as described previously (10). Hormone incubations were terminated by rapid aspiration of the bathing medium and the addition of 2 ml of ice-cold 5% TCA. 1 ml of the supernatant achieved after centrifugation was then applied to a Dowex-50 column (0.5 × 8 cm; Dow Chemical Co., Midland, MI) and eluted with 0.05 M HCl. The eluate was collected, and its cyclic AMP content assessed using a competitive binding assay (4). Using the above procedure a minimum of 90% of the total cyclic AMP was detected.

DNA Assay

The DNA content of the cultures was measured as described by Kissane and Robins (24). The dishes were rapidly rinsed twice at room temperature with physiological saline and the cells removed with a silicone rubber stopper. The samples were then ultrasonicated and quadruplicate 50-μl aliquots were transferred to tubes that were previously rinsed with double-glass distilled water. These were evaporated to dryness and incubated at 60°C for 45 min with 50 μl of 3,5-diaminobenzoic acid. The reaction was stopped with 2.5 ml of 1 M HCl. 0.5–4.0 μg of calf thymus DNA (Sigma Chemical Co.) was used to produce a standard curve.

Creatine Phosphokinase Assay

The cells were stored at −80°C until required for analysis. The plates were then rapidly thawed and the cells scraped off the dishes into cold 100 mM Tris-HCl (pH 6.8) with 5 mM magnesium acetate (BDH Chemicals, Poole, England). The resulting suspension was ultrasonicated and the creatine phosphokinase levels assayed (8, 26). A standard curve was constructed for creatine over a concentration range of 100–400 nM and subjected to the same conditions of incubation and analysis.

Visualization of the Muscle-specific Form of Creatine Phosphokinase Using Immunofluorescent Staining

The cells were fixed in −20°C acetone/methanol (1:1) for 1.5 min and stored at 4°C. Before the first antibody addition, each dish was preincubated for 30 min at 37°C with 1 ml of PBS containing 10% horse serum. The samples were then rinsed and incubated for 1 h at 37°C in >95% humidity with (a) anti–muscle-specific creatine phosphokinase at a dilution of 1/100 (generously donated by J. C. Perriard, Institute for Cell Biology, Swiss Federal Institute of Technology, Zürich, Switzerland), and (b) anti–rabbit FITC-conjugated immunoglobulin G (Sigma Chemical Co.) at a dilution of 1/40. Finally, each dish was thoroughly rinsed with PBS (pH 7.4). The cells were counted (×300) on a Zeiss III RS microscope with epifluorescence. Each nucleus was identified using phase optics and the cytoplasm immediately around the nucleus scored for positive staining with fluorescein. A tally was maintained of the total number of cells and those that stained for muscle-specific creatine phosphokinase. Percentage staining was calculated as the number of nuclei with fluorescence in their surrounding cytoplasm, divided by the total number of nuclei observed, and multiplied by 100. As this procedure involves a subjective element, the dishes were counted blind.

Assessment of DNA and Protein Synthesis

0.5 μCi per 35-mm dish of [³H]thymidine (Amersham International, Amersham, Buckinghamshire, England), made up to 10⁻⁶ M with unlabeled thymidine, or 0.5 μCi per 35-mm dish of carrier-free [³H]leucine (specific activity 60 Ci mM⁻¹) (Amersham International) were added to the dishes 6 h before harvesting. The cells were then rinsed rapidly three times with physiological saline at room temperature and removed with a silicone rubber stopper in 1 ml of physiological saline containing 1% BSA (Sigma Chemical Co.). 1 ml of 10% TCA (Sigma Chemical Co.) was added and the sample left overnight at 4°C. The resulting precipitate was centrifuged at 900 g for 5 min, the supernatant discarded, and the pellet resuspended in 2 ml of 5% TCA. This rinsing procedure was repeated twice. The final precipitate was dissolved in a scintillation cocktail (NE260; Nuclear Enterprises Limited, Edinburgh, Scotland) and counted in a liquid scintillation counter (model L5 7500; Beckman Instruments Inc., Fullerton, CA).

Table I. Inhibitions of Myoblast Fusion Achieved by Chloroquine and Indomethacin in Four Individual Experiments

Percent fusion			(Cell Nos./cm^2 dish) × 10^{-3}		
Control	Chloroquine	Indomethacin	Control	Chloroquine	Indomethacin
43.6 ± 1.6	16.3 ± 1.2	20.7 ± 1.2	34.9 ± 1.9	36.0 ± 3.5	38.7 ± 4.8
43.9 ± 1.1	16.2 ± 1.2	21.6 ± 1.0	37.7 ± 2.6	35.9 ± 3.9	37.6 ± 2.5
45.0 ± 1.7	14.5 ± 1.8	20.7 ± 1.1	42.7 ± 2.9	41.8 ± 3.7	40.3 ± 5.5
42.2 ± 3.4	14.7 ± 2.4	18.3 ± 2.0	50.9 ± 5.6	52.3 ± 9.7	52.3 ± 4.8

The data were collected from 58-h cultures. All values represent the mean of four dishes ± the SD.

Acetylcholine Receptor Assay

Acetylcholine receptors were assayed as described previously (27). 4 nCi/35-mm dish of [^{125}I]αbungarotoxin (generous gift of S. Bevan, Department of Zoology, University College London, London), specific activity 200 Ci mM^{-1}, was added to the dishes for 1 h. The cells were rinsed rapidly five times and then a further five times at 3-min intervals with Eagle's minimum essential medium (Gibco) containing 1% BSA (Sigma Chemical Co.). The cells were trypsinized with 0.7 ml of 0.04% trypsin (Difco Laboratories Inc., Detroit, MI) in calcium and magnesium free Earle's salts and the reaction stopped with 1.4 ml of Earle's salts containing 1% BSA. The harvested cells were counted in a gamma counter (model NE 1600; Nuclear Enterprises Limited).

Results

The freshly isolated cells were seeded at a density of 1.4 × 10^5/35-mm dish and exposed to a complete medium change 24 h after plating. Under these conditions, 90% of all cell fusion occurred between 48 and 68 h, with 70-75% of the total population undergoing fusion. The cells were routinely fixed in midfusion (58 h), when 40-50% of cells in control cultures had fused (Table I).

3 × 10^{-6} M Indomethacin and Chloroquine Inhibit Myoblast Fusion but Not Other Aspects of Differentiation

Before using prostanoid-synthesis antagonists as inhibitors of myoblast fusion, it was necessary to determine appropriate dosing regimes. To this end, the inhibitions of fusion achieved by indomethacin and chloroquine using 24-, 12-, and 6-h dosing were compared (Fig. 1). The data reveal that if a given total amount of chloroquine is administered frequently in small doses, the maximum inhibition obtained is increased (Fig. 1 a). In contrast, the inhibition of fusion achieved by indomethacin is independent of the frequency of addition (Fig. 1 b). These findings indicate that indomethacin is stable in the muscle cells' cultures, whereas chloroquine suffers an effective loss of potency. Under our conditions of culture, the optimal inhibitory doses are a total of 3 × 10^{-6} M indomethacin or 1 × 10^{-6} M chloroquine administered every 6 h. When either of these dosing regimes was used, the onset of fusion was prevented for ~12 h. Given the technical difficulties of dosing routinely every 6 h, 12-h dosing regimes of 1 × 10^{-6} M indomethacin or 1 × 10^{-6} M chloroquine were adopted. Table I illustrates both the effectiveness and consistency of the inhibitions imposed by these dosing regimes.

Throughout the course of the experiments, indomethacin and chloroquine were without effect on total cell numbers (Fig. 2 a, Table I). The same was true for the total cellular DNA levels. For example, there was no significant difference (at the 90% level) in the mean values obtained from four separate experiments for 58-h control, indomethacin- and chloroquine-treated cultures. Table II illustrates the cellular
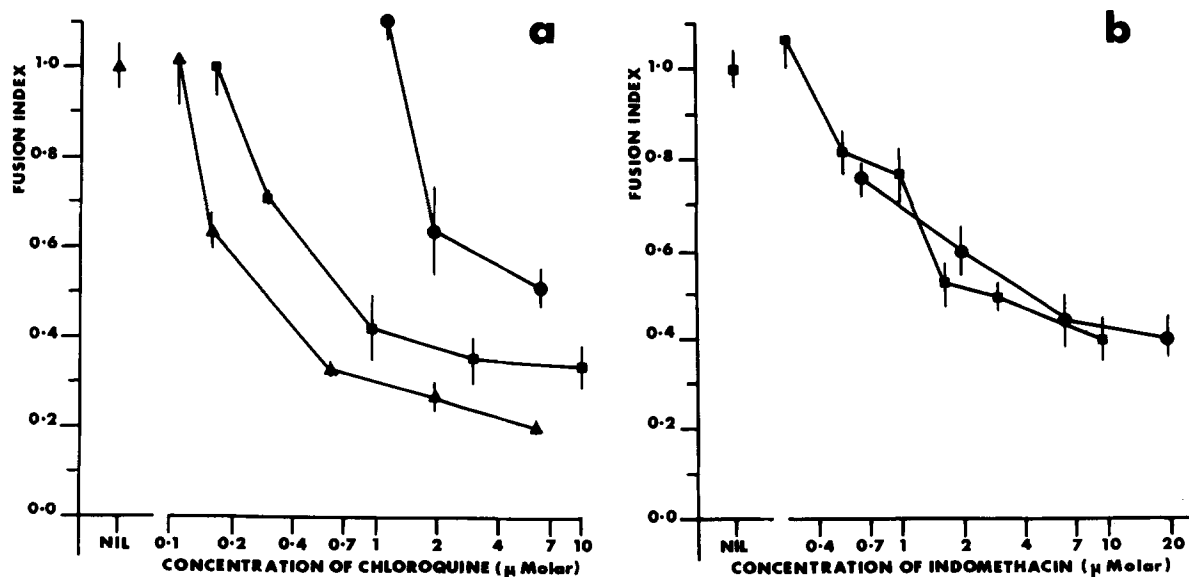

Figure 1. The effect of the frequency of dosing on the chloroquine and indomethacin inhibitions of myoblast fusion. The drugs were added from 24 h in culture at 24-h intervals (●), 12-h intervals (■), and 6-h intervals (▲). The dose response of fusion to the total drug added over the 34-h period is presented. These results were generated from three separate experiments. Each value represents the mean ± SD obtained from four separate dishes. (a) Chloroquine-treated cultures. (b) Indomethacin-treated cultures.
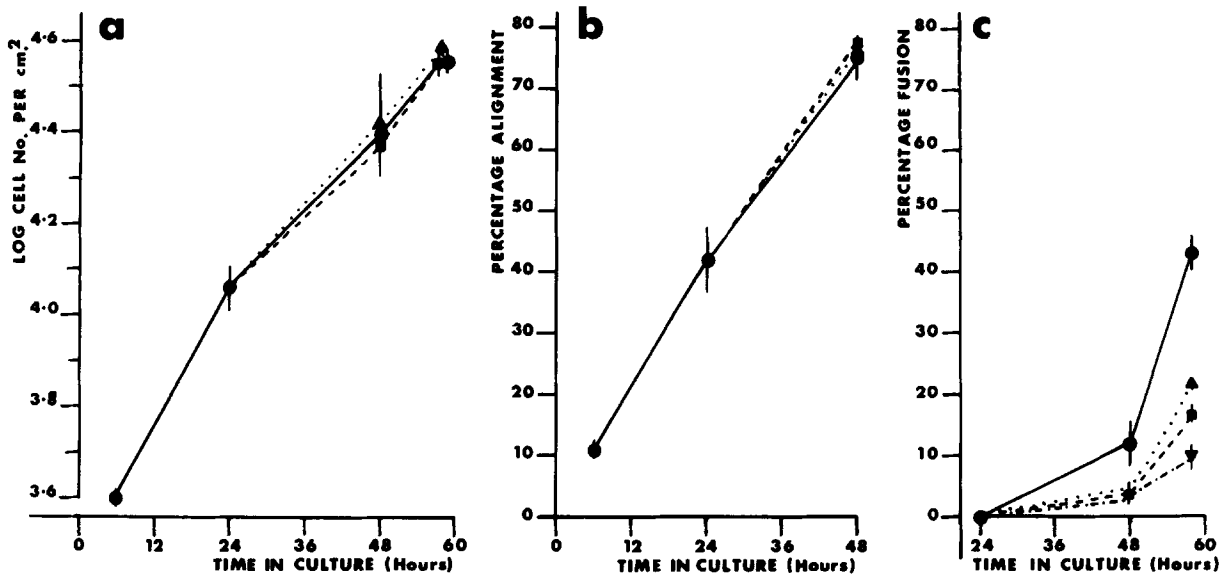
Figure 2. The time course of cell proliferation, alignment, and cell fusion under control conditions, and in the presence of indomethacin and chloroquine. Controls (●); plus 1 × 10⁻⁶ M indomethacin added every 12 h from 24 h in culture (▲); plus 1 × 10⁻⁶ M chloroquine added every 12 h from 24 h in culture (■); plus both indomethacin (1 × 10⁻⁶ M) and chloroquine (1 × 10⁻⁶ M) added every 12 h from 24 h in culture (▼). All values represent the mean of four dishes ± the SD. (a) Proliferation. Analysis of the control and drug-treated cultures revealed that the cell numbers were not significantly different at the 95% level at either 48 or 60 h. (Bartlett's test and analysis of variance). (b) Cell alignment. The small number of nuclei present in myotubes by 48 h were included in the aligned counts, allowing a consistency to be maintained between the proportion of cells that had undergone alignment in control and drug-treated cultures. The 48-h data from the control and drug-treated cultures were not significantly different at the 95% level (Bartlett's test and analysis of variance). (c) Cell fusion. The 58-h data from the control and drug-treated cultures were significantly different at >99% level (Bartlett's test, no significant difference; analysis of variance and Scheffe's multiple comparisons: all >99%).

DNA levels obtained from a typical individual experiment. As a further and more sensitive assessment of the myoblast cell cycle, the incorporation of [³H]thymidine into cellular DNA was examined. Again no difference was observed between control and treated cultures (Table II). The absence of any effect of either 3 × 10⁻⁶ M indomethacin or chloroquine on three separate parameters of cell proliferation demonstrates that myoblast proliferation is unaltered by either drug.

To fuse, myoblasts must first align (25). It was therefore

necessary to establish whether or not the drug regimes to be used had any effect on the alignment process. The numbers of aligned cells were compared in control and treated cultures by counting the total number of nuclei and those in aligned or fused cells. The mean values derived from three experiments for the proportion of nuclei in aligned/fused cells were not significantly different between control and treated cultures at the 90% level. The results of one such typical experiment are given in Fig. 2 b. The results demonstrate that from 24 to 48 h neither drug observably hinders cell

Table II. Effects of Chloroquine and Indomethacin on Myoblast Cellular Function and Differentiation

Treatment	Controls 24 h	Controls 58 h	Chloroquine 58 h	Indomethacin 58 h
% Fusion	None detected	47.9 ± 2.2	14.4 ± 2.6	20.3 ± 3.0
DNA (ng/cm² of dish)	74 ± 7	209 ± 18	195 ± 21	209 ± 11
Cell Nos./cm² of dish (× 10⁻³)	14.6 ± 2.7	48.9 ± 8.6	52.6 ± 7.1	56.3 ± 3.3
[³H]Thymidine incorporation (dpm/h)	Not determined	2,078 ± 76	2,178 ± 118	1,887 ± 188
CPK (nmol/min per ng DNA)	8.3 ± 2.0	29.0 ± 3.3	24.6 ± 3.4	29.8 ± 1.9
% M-CPK-positive staining	8.4 ± 1.5	59.5 ± 2.5	58.2 ± 4.3	57.7 ± 3.8
Bungarotoxin-binding (dpm/ng DNA)	920 ± 120	2,000 ± 160	2,350 ± 230	2,310 ± 100
[³H]Leucine incorporation (dpm/h)	Not determined	719 ± 149	795 ± 82	750 ± 12

All values represent the mean ± the SD of four dishes. CPK, creatine phosphokinase.

alignment. Other than the expected result that the treated dishes contain more aligned cells and fewer fused cells than their untreated counterparts, the two sets of cultures were in fact morphologically indistinguishable. Neither drug caused an increase in intracellular vacuolation. These findings indicate that at 3×10^{-6} M indomethacin and chloroquine have no discernable effects on gross cell morphology.

To determine the nature of the inhibition of differentiation imposed by 3×10^{-6} M indomethacin and chloroquine, the expression of two muscle-specific proteins (i.e., creatine phosphokinase and acetylcholine receptor) was examined. Table II demonstrates that neither inhibitor of prostanoid synthesis prevents the initial increase in total creatine phosphokinase, seen in differentiating myogenic cultures. The mean activities of creatine phosphokinase (at 58 h of culture), derived from three separate experiments, were not significantly different at the 90% level in control and treated cultures. The increase in creatine phosphokinase activity associated with myoblast differentiation reflects both an increase in accumulation of the nonspecific isozyme (B-creatine phosphokinase) and the de novo expression of the muscle-specific form i.e., M-creatine phosphokinase (33). No significant difference in the proportion of nuclei in cells expressing M-creatine phosphokinase was obtained between control and drug-treated cultures (Table II). Similarly, indomethacin and chloroquine do not prevent the expression of a second parameter of differentiation examined, namely the increase in the number of acetylcholine receptors on the muscle cells' surface. The $[^{125}I]\alpha$bungarotoxin binding to control and drug-treated 58-h cells (in two separate experiments) was not significantly different at the 90% level. The results obtained from one of these experiments are presented in Table II. Finally, the drugs have no effect on total protein synthesis, since there is no significant difference (at the 90% level) in the $[^{3}H]$leucine incorporated into acid precipitable material of control and drug treated cultures (Table II). From these various studies we conclude that both indomethacin

and chloroquine specifically inhibit myoblast fusion without affecting other parameters of muscle differentiation.

These specific inhibitions of fusion obtained with 3×10^{-6} M indomethacin and chloroquine administered every 12 h are not complete (Fig. 2 c), and after ~58 h of culture they rapidly disappear (data not presented). An additional tenfold excess of the drugs (i.e., 10^{-5} M) administered at 56 h does not maintain the block. The degree of inhibition observed is increased by administering indomethacin and chloroquine together; however, these improved inhibitions are also rapidly lost after 58 h of culture (data not presented). These results strongly suggest that the loss of inhibition is not due to a decrease in potency of the drugs.

Reversal of the Indomethacin- and Chloroquine-imposed Inhibitions of Cell Fusion Is Rapid, Complete, and Specific to PGE₁

The previously reported blocks of fusion imposed by indomethacin or aspirin were reversed 5-6 h after exposure to PGE₁ (11, 44). In contrast, reversal of the inhibitions of fusion achieved upon returning low external calcium to control levels can be more rapid, occurring after ~1.5 h (31). The specific nature of the inhibitions of myoblast fusion imposed by chloroquine and indomethacin suggest that like the calcium reversals, the reversal by PGE₁ should be rapid. A 1.5-h exposure to PGE₁ ($0.32-1.0 \times 10^{-9}$ M) was sufficient to achieve control levels of fusion in cultures treated with either indomethacin or chloroquine (Fig. 3). A closer examination of the kinetics of the PGE₁ reversals revealed that 1×10^{-9} M PGE₁ has no effect on the gross morphology of the aligned cells for the first 8 min after its addition. Between 8 min and 1 h, difficulty is experienced in assessing the state of many of the closely opposed nuclei under the light microscope. After 1 h of exposure the morphological changes are sufficiently distinct to assess fusion once more. The fusion levels in these 1-h cultures are similar to that of controls (data
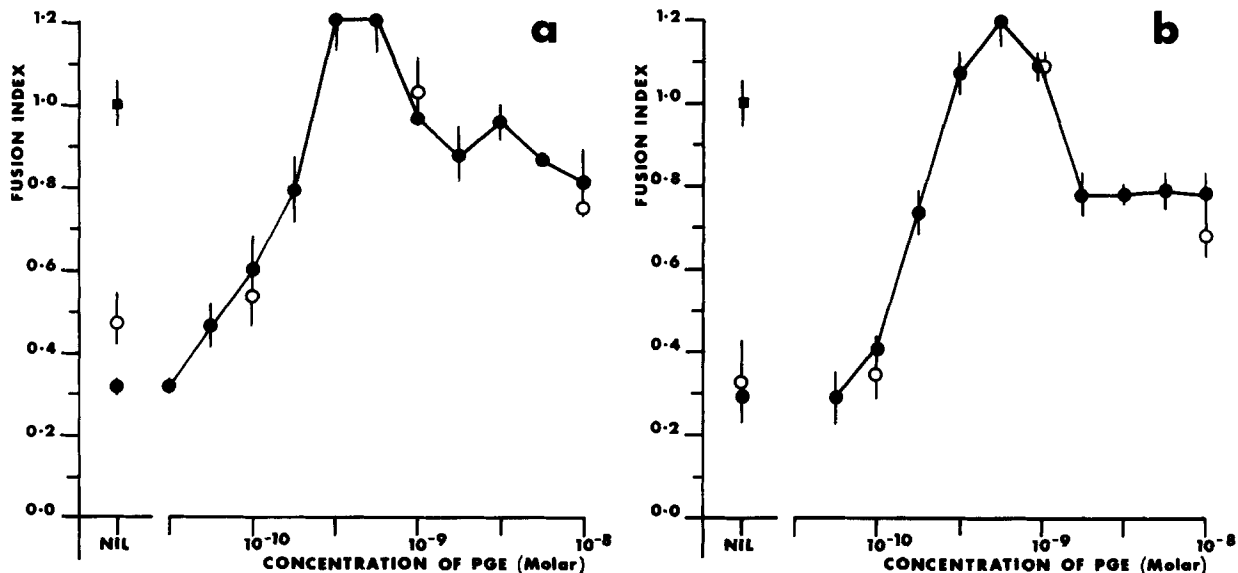

Figure 3. Fusion achieved in indomethacin- and chloroquine-treated cultures exposed to PGE₁ 10 or 1.5 h before fixation. PGE₁ addition 10 h before fixation (○); PGE₁ addition 1.5 h before fixation (●). These results were generated from four separate experiments; each value represents the mean ± SD obtained from four separate dishes. (a) Indomethacin-treated cultures. (b) Chloroquine-treated cultures.

not presented), demonstrating that the PGE$_1$ induction of fusion is rapid. Exposure of the inhibited culture to PGE$_1$ for the longer period of 10 h produced the same pattern of reversal (Figs. 4 and 5).

PGE$_1$ is the only prostanoid whose effects upon fusion have been previously examined. To determine the optimal dose for the reversal of the drug-based inhibitions of fusion and the specificity of this prostaglandin action, dose responses for the reversals by PGE$_1$ and its close relative PGE$_2$ were carried out. The prostaglandins were added to the fusion inhibited cultures at 48 h and fusion assessed 10 h later at 58 h. Fig. 4 demonstrates that PGE$_1$ but not PGE$_2$ reverses the blocks imposed on fusion by the prostanoid synthesis antagonists. No reversal of the block of fusion is achieved over a 10-h exposure of the myoblasts to a wide range of PGE$_2$ concentrations (10^{-11}–10^{-6} M). The PGE$_1$-specific response of the myoblast is unexpected since in general PGE$_1$ and PGE$_2$ are equipotent.

The dose-response curves obtained for the PGE$_1$ reversal are bell-shaped. At concentrations of PGE$_1$ higher than those necessary to elicit complete reversals of either of the drug-imposed inhibitions of fusion ($3.2 \times 10^{-10} - 1 \times 10^{-9}$ M), additional effects are observed. Concentrations of PGE$_1$ in excess of 3.2×10^{-9} M generate only partial reversals of the inhibitions imposed by either indomethacin (Figs. 3 and 4) or chloroquine (Figs. 3 and 5). This result, which reveals that higher concentrations of the prostaglandin are antagonistic to fusion, is independent of whether the cultures are exposed to PGE$_1$ for 1.5 or 10 h. Similar levels of fusion (i.e., 70% of untreated) are also obtained in control cultures exposed to concentrations of PGE$_1$ in excess of 10^{-8} M. Furthermore, in contrast to its ineffectiveness as a reversing agent of the inhibited cultures, PGE$_2$ at 10^{-8} M and above similarly depresses control fusion (Fig. 5). In their depres-

sion of control fusion the two prostaglandins are therefore equipotent. The similarity between the levels of fusion achieved by concentrations in excess of 10^{-8} M of PGE$_1$ and PGE$_2$ in control cultures and of PGE$_1$ in the fusion-blocked cultures suggests that these actions have a common underlying mechanism. The two opposing effects of PGE$_1$, stimulatory and inhibitory, are presumably responsible for the bell shape of the fusion dose response curve achieved when the prostaglandin is added to cultures previously treated with either indomethacin or chloroquine (Figs. 3–5).

Effects of Prostanoid Precursors on Fusion in Indomethacin- and Chloroquine-treated Cultures

To further investigate the specificity of the prostanoid reversal of the inhibitions of cell fusion, the effects of the prostanoid precursor fatty acids eicosatrienoate, arachidonate, and linoleate were assessed. Eicosatrienoate and arachidonate are direct precursors for prostanoids of the one and two series, respectively. Linoleate is an indirect precursor of the one series prostanoids since eicosatrienoate is generated from this fatty acid as a consequence of Δ-6 dehydrogenase activity (32). Both eicosatrienoate and linoleate give complete reversals of the inhibitions of fusion imposed by either indomethacin (Fig. 6) or chloroquine (Fig. 7). Arachidonate, the precursor to the two series, fails to reverse the drug-based inhibitions over a wide range of concentrations (10^{-11}–10^{-6} M). Because all three fatty acid esters should be converted to prostanoids, this result suggests that reversal of the inhibitions is only achieved when prostanoids of the one series are generated. The dose-response curves for reversal of the chloroquine-imposed block by eicosatrienoate, linoleate, and PGE$_1$ are very similar. In all cases a bell-shaped curve is generated with complete reversal at 10^{-9} M, and
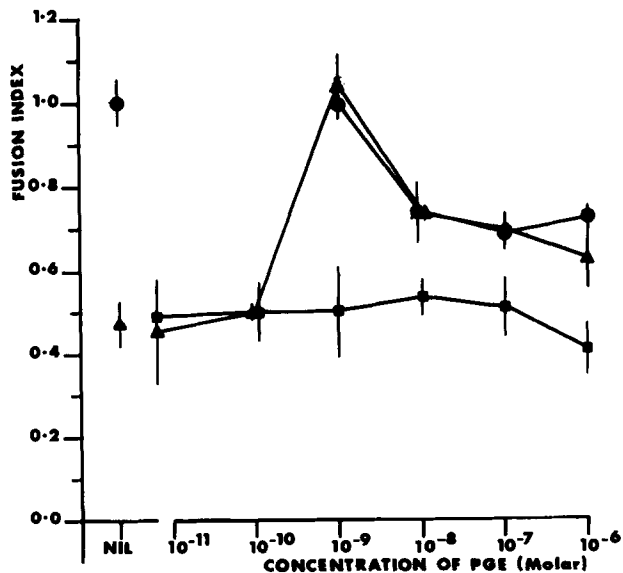
Figure 4. Fusion achieved in control and indomethacin-treated cultures exposed to different concentrations of PGE$_1$ or PGE$_2$. Control plus PGE$_2$ (●); 1 × 10^{-6} M indomethacin (dosed every 12 h from 24 h in culture) plus (▲) PGE$_1$; 1 × 10^{-6} M indomethacin plus PGE$_2$ (■). The results were generated from two separate experiments; each value represents the mean ± SD obtained from four separate dishes.
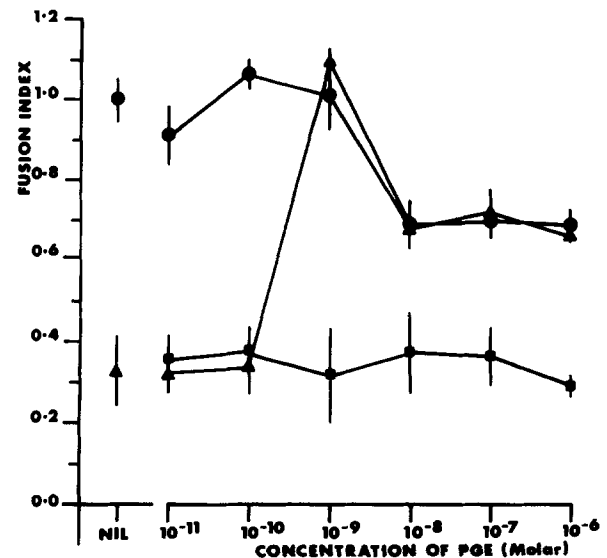
Figure 5. Fusion achieved in control and chloroquine-treated cultures exposed to different concentration of PGE$_1$ or PGE$_2$. Control plus PGE$_1$ (●); 1 × 10^{-6} M chloroquine (dosed every 12 h from 24 h in culture) plus PGE$_1$ (▲); 1 × 10^{-6} M chloroquine plus (■) PGE$_2$. The results were generated from two separate experiments; each value represents the mean ± SD obtained from four separate dishes.
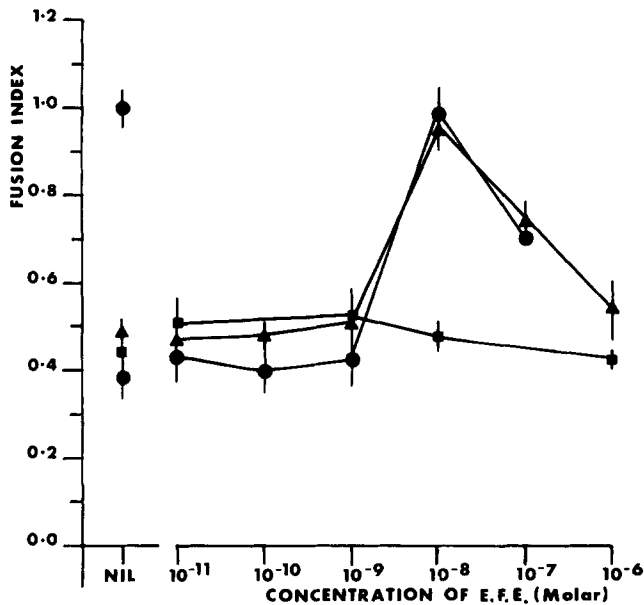
Figure 6. Fusion achieved in indomethacin-treated cultures exposed to different concentrations of the precursor essential fatty acid esters of PGE_1 and PGE_2. 1×10^{-6} M indomethacin dosed every 12 h from 24 h in culture; plus eicosatrienoate (▲); plus linoleate (●); plus arachidonate (■). These results were generated from three separate experiments; each value represents the mean ± SD obtained from four separate dishes. E.F.E., essential fatty acid ester.
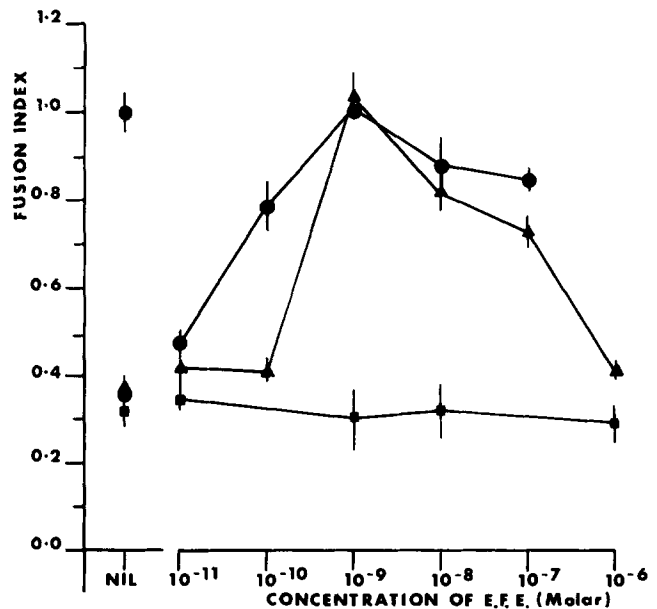

Figure 7. Fusion achieved in chloroquine-treated cultures exposed to different concentrations of the precursor essential fatty acid esters of PGE_1 and PGE_2. 1×10^{-6} M chloroquine dosed every 12 h from 24 h in culture, plus eicosatrienoate (▲); plus linoleate (●); plus arachidonate (■). These results were generated from three separate experiments; each value represents the mean ± SD obtained from four separate dishes. E.F.E., essential fatty acid ester.

lesser degrees of reversal at higher concentrations. The indomethacin block of cell fusion is also reversed by both linoleate and eicosatrienoate and bell-shaped curves are again generated. In this case the doses required for maximal and partial reversals are both 10-fold higher than those effective for the reversal of the chloroquine-treated cultures.

Reversal of the chloroquine and indomethacin inhibitions by linoleate and eicosatrienoate has an important implication when considering experimental reproducibility. If the levels of these essential fatty acids in the culture medium are high, they will clearly reduce the effectiveness of prostanoid synthesis inhibitors. That this is a factor in determining the inhibitions obtained is suggested by our observation that the batch of serum employed markedly affects the degree of inhibition achieved by both indomethacin and chloroquine. Similar variability with serum batch has been observed in the inhibition of rabbit platelet aggregation by the chloroquine analogue mepacrine (2).

These results demonstrate that the inhibitions of fusion generated by either indomethacin or chloroquine can be overcome by the appropriate precursor fatty acids. Chloroquine most probably acts by reducing the availability of free fatty acid, whereas indomethacin prevents the conversion of the fatty acid to prostanoids. Given their different sites of action, it can be predicted that indomethacin and chloroquine will act synergistically. Such a synergism is indeed seen. When indomethacin and chloroquine are administered together, an increased inhibition of 86% is obtained (Fig. 8). This inhibition achieved with indomethacin and chloroquine combined (3×10^{-6} M) is completely reversed by 10^{-9} M PGE_1 (data not presented).
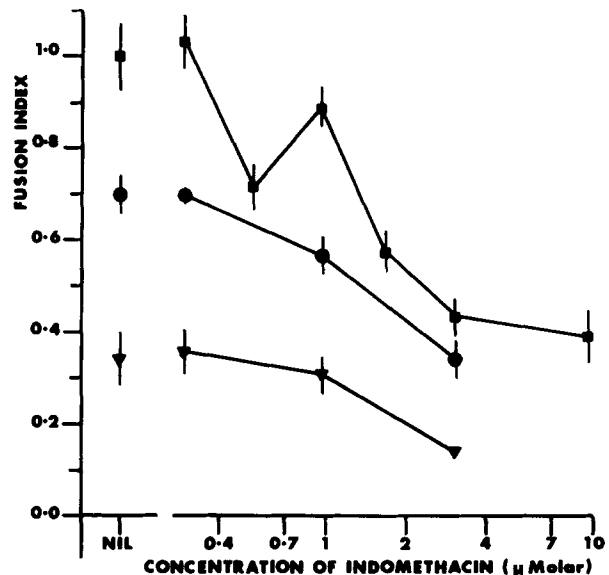

Figure 8. The dose response of fusion to the total indomethacin added with different levels of chloroquine present. Both indomethacin and chloroquine were added every 12 h from 24 h in culture. Indomethacin alone (■); indomethacin plus 1×10^{-7} M chloroquine (●); indomethacin plus 1×10^{-6} M chloroquine (▲). The results were generated in a single experiment; each value represents the mean ± SD obtained from four separate dishes.

PGE_1 Cyclic AMP and Cell Fusion

PGE_1 raises intracellular cyclic AMP in avian muscle cell cultures (11, 29). However, at concentrations that completely reverse the inhibitions of fusion imposed by 3×10^{-6} M indomethacin or chloroquine, PGE_1 has no effect on the intra-

Table III. Effects of Various Concentrations of PGE_1 and Isoproterenol on Myoblast Fusion and Intracellular Cyclic AMP

Treatment	Fusion index	Fold increase in cyclic AMP
Control	1.00 ± 0.05	1.00 ± 0.25
PGE_1 (10^{-9} M)	1.07 ± 0.08	0.93 ± 0.22
PGE_1 (10^{-7} M)	0.70 ± 0.02	2.52 ± 0.27
Isoproterenol (10^{-5} M)	0.70 ± 0.04	2.32 ± 0.33
Isoproterenol (10^{-5} M) plus propranalol (5×10^{-5} M)	0.99 ± 0.01	1.03 ± 0.05
Isoproterenol (10^{-8} M)	0.99 ± 0.06	1.47 ± 0.20
Dibutyryl cyclic AMP (1×10^{-4} M)	0.67 ± 0.06	Not determined

Fusion index values represent the mean of four dishes $\pm$ the SD. Fold increase in cyclic AMP values represent the mean of four sets of duplicate values $\pm$ SD.

cellular cyclic AMP levels (Table III). In agreement with the previous studies, cyclic AMP is increased by PGE_1, but this only occurs when concentrations of the prostaglandin are reached that inhibit control myoblast fusion (Table III). Isoproterenol, a synthetic β-adrenergic agonist, also raises cyclic AMP in avian myoblast cultures (9). At the concentrations of isoproterenol that effectively increase cyclic AMP, it too inhibits control cell fusion under our conditions (Table III). The addition of the β-adrenergic-blocker propranolol 2 min before isoproterenol prevents both the depression of cell fusion and the increase in intracellular cyclic AMP (Table III). These findings suggest that increases in intracellular cyclic AMP of the order of 200–300% (assayed in the presence of isobutylmethylxanthine) can inhibit myoblast fusion. Confirmation of this is provided by the similar depressions of control fusion achieved in cultures exposed to dibutyryl cyclic AMP (26, 36, Table III).

Discussion

Indomethacin and Chloroquine Impose a Specific Block upon Myoblast Fusion

The data presented here demonstrate that indomethacin and chloroquine at 3×10^{-6} M inhibit myoblast fusion without affecting either the expression of acetylcholine receptors or the muscle-specific form of creatine phosphokinase. The results also reveal the lack of effect of either agent on the overall level of protein synthesis, cell numbers, and cell alignment. Thus, with the dosing regimes employed here, indomethacin and chloroquine act by increasing the ratio of bipolar to multinucleate cells without any other discernable effects on the morphology, proliferation, or differentiation of the myogenic cells.

In addition to their high degree of specificity with respect to myoblast fusion, the inhibitors exhibit further properties that make them very useful tools in the examination of the mechanisms involved in myoblast fusion. First, the inhibitions of fusion imposed by either drug are completely and rapidly reversible. Second, providing that the inhibitions of fusion imposed by indomethacin and chloroquine are studied at times corresponding to the early stage of fusion in the controls, they are both consistent and reproducible.

Myoblasts that have been prevented from fusing by either

indomethacin or chloroquine are induced to fuse by PGE_1. The simplest explanation for this observation is that indomethacin and chloroquine interfere with either the synthesis or action of fusion-triggering factors. Indomethacin is best known as an inhibitor of cyclooxygenase (16, 38) and consequently prostanoid synthesis. Other reported effects of the drug, with one exception, are seen only at concentrations significantly higher than those employed in the present study (16, 36). The exception to this is the inhibition of cyclic AMP-dependent protein kinase by 10^{-7} M indomethacin (21). Since we find that increased cyclic AMP is inhibitory to fusion, it is unlikely that blockage of cyclic AMP-dependent protein kinase accounts for the observed inhibitions of fusion.

Chloroquine inhibits prostanoid synthesis by blocking phospholipases (20, 28, 29, 35), the major enzymes responsible for the de-esterification and release of the essential fatty acids from which the prostanoids are synthesized (17, 19). It has also been demonstrated to inhibit cell replication, protein synthesis, and lysosomal function (6, 7, 13). In this study chloroquine has no effect on DNA synthesis, cell numbers, or protein synthesis. It is therefore unlikely that either of the first two alternatives represents its critical action with respect to cell fusion. At higher concentrations of chloroquine than those used in the present study, the drug inhibits M-creatine phosphokinase synthesis, in common with other lysosomotropic agents (22). At such concentrations it also causes extensive intracellular vacuolation (unpublished observation) which is a general characteristic of lysosomotropic action (15). These effects on creatine phosphokinase and intracellular vacuolation were not observed at the lower concentrations of chloroquine used to specifically block fusion, making it unlikely that extensive interference of lysosomal function is occurring. Additionally, interference with lysosomal function may also reflect chloroquine's inhibition of phospholipases (35). We therefore conclude that in this study the effects of indomethacin and chloroquine are wholly consistent with their interfering with the action of an endogenously produced fusion-regulating prostanoid.

A Prostanoid of the One Series Is Involved in the Regulation of Myoblast Fusion

Cyclooxygenase does not select between its two substrates, and both arachidonate and eicosatrienoate are rapidly converted to the corresponding prostaglandins (42). The inability of arachidonate to reverse the fusion block therefore implies that it is only prostanoids belonging to the one series which can trigger fusion. It is unlikely that eicosatrienoate and linoleate act directly to trigger fusion, since these precursor fatty acids would then mimic PGE_1. PGE_1 generates the same dose responses for the reversal of both indomethacin and chloroquine; this is not the case with eicosatrienoate or linoleate (Figs. 4 and 5 compared with Figs. 6 and 7). The finding that a prostanoid of the one series is a specific effector of myoblast fusion is unexpected and has a number of implications. First, although in many of their actions PGE_1 and PGE_2 are equipotent, the PGE_1- and eicosatrienoate-specific effects reported here imply that the myoblast possesses a receptor with restricted sensitivity to prostaglandins of the one series. Second, cell membranes rarely contain significant quantities of esterified eicosatrienoate (32). This is also true of the myoblast (3, 23). This

raises the question of the source of the eicosatrienoate. One possibility is linoleate. Myoblast membranes contain large quantities of this fatty acid in esterified form. Furthermore, in many tissues and cell types, it has been demonstrated that eicosatrienoate can be generated from linoleate by the action of Δ-6 dehydrogenase (32). That this is the case in the myoblast is suggested by the ability of linoleic acid to stimulate control fusion (34) and to be as effective as eicosatrienoate in overcoming the inhibitions imposed upon fusion by indomethacin and chloroquine. We conclude therefore that the lack of esterified eicosatrienoate does not preclude the involvement of a series-one prostanoid in the regulation of myoblast fusion.

Finally, the transient nature of the indomethacin and chloroquine-based inhibitions of fusion must be considered. Adding higher concentrations of these drugs (1×10^{-5} M) before the loss of inhibition of fusion at $\sim$58 h of culture does not prolong the block imposed by either of the drugs. This suggests that the loss of these inhibitions is not due to a loss in efficacy of the inhibitors of prostanoid synthesis. It further implies that during fusion, a prostanoid-independent mechanism is operating to initiate fusion. That this is so is indicated by the finding that fusion is also controlled through the acetylcholine receptors (1) utilizing an as yet unidentified agonist. From these findings we conclude prostanoids are not the only regulators of myoblast fusion.

Cyclic AMP Does Not Mediate the Prostanoid Regulation of Fusion

It seems very unlikely that cyclic AMP is the mediator of PGE₁-induced fusion. At the concentrations of the prostaglandin that completely reverse the drug-imposed inhibitions of cell fusion, PGE₁ does not significantly raise intracellular cyclic AMP. Additionally, at the higher concentrations of PGE₁ capable of increasing the level of the cyclic nucleotide, control fusion is depressed and only partial reversals of the block imposed by the inhibitors of prostanoid synthesis are achieved. From these observations, plus the ineffectiveness of the β-adrenergic agonist isoproterenol to reverse the fusion block, we conclude that PGE₁ does not trigger fusion by activation of adenylate cyclase. The results in fact indicate that increases in cyclic AMP are antagonistic to the fusion event.

The apparent contradiction between the cyclic AMP depression of fusion seen here and previous reports of stimulations of cell fusion by the cyclic nucleotide is reconcilable. The stimulations of myoblast fusion by cyclic AMP occur when the synthesis of the cyclic nucleotide is induced $\sim$10 h before the predicted time of the onset of fusion in controls (9, 44). The depression of fusion reported here is the consequence of exposing myoblasts that are undergoing active fusion to an increase in cyclic AMP. There is also a difference in the time it takes the cell to respond in the two situations. A minimum lag of 4–5 h is seen before an elevation of cyclic AMP induces precocious fusion, whereas the inhibitions of fusion are imposed by the cyclic nucleotide within 1.5 h. Taken together, these findings have two implications. First, that the depression of fusion by cyclic AMP is a separate action from the cyclic–nucleotide-mediated stimulation of cell fusion. Second, that the depressions of fusion imposed by cyclic AMP are due to the cyclic nucleotide's antagonism of a process that is occurring close to the fusion event itself.

The obvious alternative as intracellular mediator of prostanoid triggered cell fusion is calcium. The dependence of myoblast recognition–alignment on extracellular calcium is well documented (25). In addition, there is evidence that calcium is required for the fusion event itself. For example, the calcium ionophore A23187 induces precocious fusion in control cultures (11, 12), whereas the calcium blockers D600 and lanthanum prevent its onset (11, 14). In a preliminary study to explore the possible connection between extracellular calcium and PGE₁ action, we have found that both D600 and lanthanum prevent the prostaglandin reversal of the fusion block imposed by inhibitors of prostanoid synthesis (14). This result implies that PGE₁ triggers fusion by causing an influx of calcium, a contention that is supported by the following findings. First, PGE₁ increases the net influx of calcium into fusing chick myoblasts. Second, the kinetics of the fusion achieved after the return of external calcium levels to normal levels or the addition of PGE₁ to fusion-blocked cultures are very similar. Taken together, the data strongly suggest that myoblast fusion is regulated by a series-one prostanoid that triggers an influx of calcium. Work is now in progress to elucidate the mechanism of calcium entry.

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