ential plot was best suited for determining the resolution limit of agreement in reciprocal space and for assessing the relative merits of different alignment strategies. Above 1/150 $Å^{-1}$, the phase residual decreases substantially in the refinement cycles, whereas it shows the opposite behavior at and below 1/150 $Å^{-1}$. This behavior indicates that in going from the first to the second alignment cycle, accuracy in the alignment of strongly variable, low-resolution features of the outer stain layer is traded off for an increased accuracy in the alignment of high-resolution features.

By allowing a maximum phase residual of 45°, we obtain from Fig. 3a a limiting resolution of 1/32 $Å^{-1}$. Indeed, the total average of Fig. 2a when limited to this resolution (Fig. 2b) still shows the prominent features observed in the unfiltered average.

The phase residual was also used to check the dependence of the result on the choice of the initial reference. We repeated the entire three-cycle alignment procedure with a different particle as reference and determined the phase residual of averages over the same particle subset (Fig. 2c) obtained with the two different reference particles. The values of the phase residual (Fig. 3a) indicate that the agreement is closer than the agreement between averages over different subsets obtained with the same reference particle. This result is to be expected for the limited number of particles used.

A plot of the variance as a function of the number of images $N$ averaged allows the gain in signal-to-noise ratio (s/n) to be determined (*14*). The variance of the average image, $\sigma^{(N)^2}$, is expected to decrease according to

$$\sigma^{(N)^2} = \sigma^2 + \frac{1}{N}\sigma_n^2 \qquad (4)$$

where $\sigma^2$ is the variance of the signal common to the image set and $\sigma_n^2$ the variance of the noise, modeled as Gaussian and additive. Plotting $\sigma^{(N)^2}$ as a function of $1/N$ shows an approximately linear dependence above $N = 6$ (Fig. 3b). From the slope and intersection of the straight line with the variance axis ($N = \infty$), we could calculate the average initial s/n, $\sigma^2/\sigma_n^2 = 0.55$, and the residual s/n in the average of all particles (Fig. 2a), $(N\sigma^2)/\sigma_n^2 = 42.4$.

The reproducibility of the density distribution obtained by single-particle averaging approaches that of maps obtained by Fourier averaging of micrographs of ordered protein arrays [see, for example (*15*)]. In this range of accuracy, rounding errors and errors of interpolation in the digital analysis must be seriously considered.

The approach of single-particle averaging rests on the assumption that all of the images are comparable. However, interparticle variations, which will tend to blur the averaged image, arise from several sources. The strength of the outer stain layer varies relative to the mean density of the stain-excluding regions. The effective tilt angle may also vary. The lateral view analyzed may, in fact, comprise a small range of tilted views of the 40*S* subunit, with the tilt axis likely parallel to the particle's long axis.

Greater homogeneity of the image set to be averaged—and thus finer resolvable detail—can be obtained through application of multivariate statistical analysis (*16*). Even without this refinement, the realization of a quantitatively reproducible, averaged projection is a major step toward a high-resolution, three-dimensional model of the ribosome structure.

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17. We thank P. N. T. Unwin for a critical reading of the manuscript and D. DeRosier for discussions.

1 June 1981; revised 23 September 1981

## Regulation of Muscle Differentiation: Stimulation of Myoblast Fusion in vitro by Catecholamines

Abstract. *Epinephrine and isoproterenol provoke primary chick myoblasts to initiate precocious cell fusion. Both the rise in intracellular adenosine 3',5'-monophosphate (cyclic AMP) and cell fusion generated by these effectors are prevented by propranolol, which is a specific blocker of the β-adrenergic receptor. Propanolol has no effect either on the precocious cell fusion provoked by prostaglandin E or on cell fusion in control cultures. The results support the idea that a rise in cyclic AMP is the critical intracellular change responsible for initiating events that culminate in myoblast differentiation 4 to 5 hours later. They also indicate that the hormone responsible for the positive regulation of myoblast differentiation in vitro is not acting through the β-adrenergic receptor.*

Myoblast differentiation involves the fusion of cell membranes to form the postmitotic multinucleate units characteristic of adult skeletal muscle tissue and the concomitant elaboration of muscle-specific proteins such as the acetylcholine (ACh) receptor, the contractile proteins, and the muscle-specific form of creatine phosphokinase. The initial stages of muscle differentiation do not depend on changes that result from cell fusion (*1–4*). Nevertheless, the temporal correlation between the appearance of some muscle-specific products and myoblast fusion (*5*) suggests the existence of a coordinating regulatory mechanism. A central question related to muscle differentiation is therefore the nature of the control mechanisms that regulate the transition from the proliferating single cell to the differentiated postmitotic multinucleate unit and that coordinate the appearance of the different aspects of the cells' differentiated state.

Several lines of evidence suggest that cyclic nucleotides play an important role in the regulation of muscle differentiation. First, a transient rise in intracellular adenosine 3',5'-monophosphate (cyclic AMP) occurs shortly before the onset of


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Table 1. The effect of propranolol (PR) on myoblast fusion in control cultures and those exposed to either PGE or isoproterenol (IP). Propranolol at $3 \times 10^{-5}M$ and PGE and IP at $1 \times 10^{-5}M$ were added at 34 hours of culture; vehicle (Earles salts) alone was added to the controls.

| Addition | Number of nuclei present between 40 and 53 hours of culture | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 40 | | 44 | | 48 | | 53 | |
| | Total | Myotube | Total | Myotube | Total | Myotube | Total | Myotube |
| None | 771 ± 93* | 49 ± 8* | 645 ± 27* | 50 ± 19* | 832 ± 122* | 132 ± 56* | 1086 ± 23† | 275 ± 2† |
| PR | | | | | 748 ± 54 | 140 ± 67 | 973 ± 61 | 233 ± 18 |
| IP | 671 ± 94 | 87 ± 28 | 742 ± 34 | 120 ± 24 | 869 ± 82 | 208 ± 40 | 1062 ± 42 | 378 ± 9 |
| PR + IP | 626 ± 54 | 47 ± 11 | 616 ± 66 | 53 ± 19 | 806 ± 40 | 79 ± 7 | 954 ± 6 | 236 ± 9.6 |
| PGE | 695 ± 80 | 107 ± 19 | 796 ± 113 | 134 ± 29 | 869 ± 72 | 228 ± 28 | | |
| PR + PGE | 900 ± 131 | 144 ± 20 | 845 ± 82 | 142 ± 10 | 935 ± 92 | 186 ± 9 | | |

*Mean ± standard deviation of three experiments. †Mean of two experiments ± one-half the duplicate difference.

chick myoblast fusion (*6*). Second, exposure of the undifferentiated myoblasts to prostaglandin E (PGE) provokes a similar increase in cyclic AMP and results in precocious myoblast fusion (*7, 8*). Third, agents (including PGE) that raise intracellular cyclic AMP increase the numbers of ACh receptors that are incorporated into differentiating myotube membranes (*9*). These observations are consistent with our proposal that it is a rise in cyclic AMP that triggers the intracellular events that culminate in the expression of both of these aspects of muscle differentiation (*6, 8*). However, they do not rule out the possibility that some other intracellular change or changes provoked by the prostaglandin are responsible for the observed increases in myoblast fusion and ACh receptors. The experiments reported here were performed to determine whether other agents that are capable of increasing the concentration of myoblast intracellular cyclic AMP can provoke precocious myoblast fusion. Two β-adrenergic agonists were employed for this purpose.

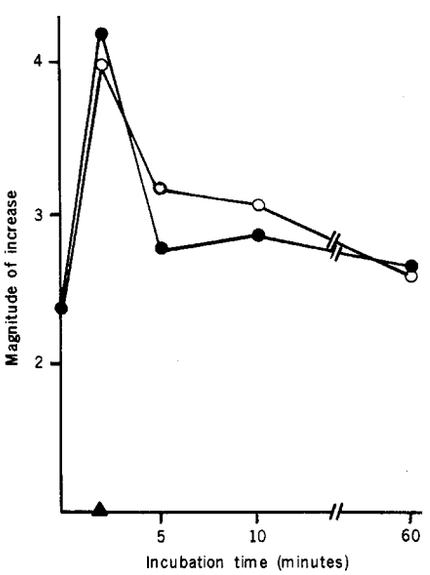


Fig. 1. The β-adrenergic stimulation of cyclic AMP formation in 32-hour chick myoblast cultures. Stimulations by $10^{-5}M$ isoproterenol (●), $10^{-5}M$ epinephrine (○), and $10^{-5}M$ isoproterenol in the presence of $3 \times 10^{-5}M$ propranolol (▲). Each value represents the average for duplicate dishes. The resting intracellular concentration of cyclic AMP was 138 pmoles per milligram of DNA.

The preparation and conditions of culture were as described in (*6*), and a seeding density of $5 \times 10^4$ cell/cm$^2$ was adopted. The ability of the β-adrenergic agonists *l*-isoproterenol and *l*-epinephrine bitartrate to induce cyclic AMP accumulation and precocious fusion was examined after their addition at 34 hours of culture. Cyclic AMP was extracted and assayed as described in (*6*). Both *l*-isoproterenol and *l*-epinephrine at $10^{-5}M$ (maximal stimulation) induce approximately twofold increases in intracellular cyclic AMP (Fig. 1). The main burst of cell fusion begins in control cultures between 44 and 48 hours (Fig. 2). In the presence of either of the hormones at $10^{-5}M$, the fusion indices at 40, 44, and 48 hours are double ($P < .05$) those in controls (Fig. 2). For comparison, the effects of PGE are included, and Table 1 shows that all three additions provoke similar numbers of cells to fuse precociously (an average of 9.5 percent of the total population). Comparable cell numbers are present in control and treated cultures at 40, 44, and 48 hours (Table 1); it is therefore improbable that the observed increase in numbers of fused cells in the treated cultures is the result of either toxic or differential effects of the hormones on the myoblast and fibroblast subpopulations.

Thus the results demonstrate that catecholamines that raise cyclic AMP by interaction with the β-adrenergic receptor are able to induce precocious cell fusion. The limitation of the response to a subpopulation of myoblasts is predictable, given the transience of the cyclic AMP increase provoked by all three additions (Fig. 1) (*8*) and what is known about myoblast behavior. With respect to the latter, it has been demonstrated that the myoblast continues to cycle until its entry into a myotube (*10, 11*). A restriction of the response to a subpopulation of the cells at any one point in time is therefore inevitable since both cell fusion (*12*) and the ability of the myoblast to respond to a cyclic AMP increase by fusing (*13*) are restricted to the $G_1$ phase of the cell's cycle. The similarity in the time course of stimulation of fusion by both the catecholamines and PGE (Fig. 2 and Table 1) is consistent with a common underlying mechanism.

The myoblasts were next exposed to *dl*-propranolol, a specific blocker of the β-adrenergic receptor, from 34 hours in culture in the presence and absence of the hormones. Our initial aim was to confirm that the ability of the catecholamines to raise cyclic AMP and to trigger precocious fusion requires that they interact with myoblast β-adrenergic receptors. At $3 \times 10^{-5}M$, propranolol prevents both *l*-isoproterenol-induced cyclic AMP accumulation (Fig. 1) and precocious cell fusion (Table 1). These findings indicate the presence of β-adrenergic receptors on the chick myoblast sur-

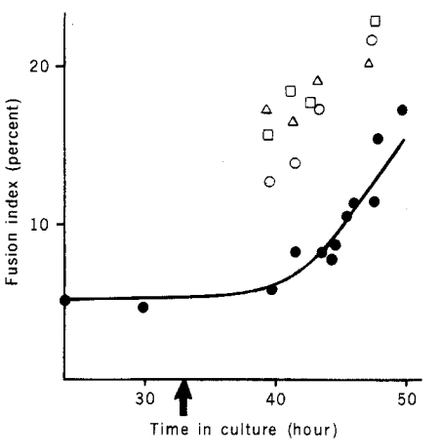


Fig. 2. Effects of β-adrenergic agents and prostaglandin E on myoblast fusion. Fusion in control (●), with $10^{-5}M$ isoproterenol (○), with $10^{-5}M$ epinephrine (△), and with $10^{-6}M$ PGE (□). Arrow indicates the time of addition of test agents. Each point represents the mean fusion index determined from at least six control cultures and three treatments.

face and suggest that the catecholamine activates adenylate cyclase specifically through this receptor. The existence of β-receptors on the surfaces of cells of the rat myoblast cell lines has been established (*14–16*). However, Parent *et al.* (*17*) were unable to detect them on quail myoblasts before differentiation. The ability of the blocker to also prevent precocious fusion supports the idea that cyclic AMP is the positive intracellular regulator.

Finally, use was made of propranolol to determine whether the activation of adenylate cyclase through the β-adrenergic receptor is an obligatory step in the induction of cell fusion by PGE. We found that cyclic AMP accumulation in response to $10^{-5}M$ PGE was normal in the presence of propranolol (*18*) and that propranolol was not able to inhibit the precocious fusion provoked by PGE (Table 1). This result demonstrates that the positive regulation of myoblast fusion in vitro by a PGE-induced rise in cyclic AMP (*7, 8*) is independent of β-adrenergic stimulation. It was still possible that under control conditions of culture (no exogenous hormone), cyclic AMP generation was dependent on activation of adenylate cyclase through the β-adrenergic receptor. (Prostaglandins are modulators of hormone action, and the positive regulation of myoblast fusion might require PGE to act in conjunction with a hormone-activating adenylate cyclase through the β-adrenergic receptor.) To test this possibility, propranolol alone was added to the undifferentiated cultures. Table 1 shows that the β-antagonist has no observable effect on either cell numbers per field or cell fusion. Thus fusion in vitro is independent of β-adrenergic activation.

This finding is in contrast to the complete inhibition of cell fusion observed in the presence of inhibitors of prostaglandin production (*4*) and supports our proposal that a prostaglandin or related molecule is the endogenous signal responsible for generating the intracellular rise in cyclic AMP in vitro. Taken together, the findings presented here are consistent with our model of myoblast differentiation (*13*). That is, a prostaglandin or related molecule is generated within the myoblast culture and interacts with a myoblast in the $G_1$ phase of its cell cycle, provoking a transient rise in intracellular cyclic AMP. The cyclic nucleotide in turn triggers the intracellular events that culminate in cell fusion. The increase of ACh receptor numbers in response to agents that raise cyclic AMP, at times when cell fusion is normally complete (*9*), suggests that this effect of cyclic AMP is not contingent on stimulation of cell fusion. Instead, the effects of cyclic AMP on cell fusion and ACh receptors are consistent with cyclic AMP acting as a simultaneous initiator of a number of independent events that represent the different aspects of the expression of muscle's differentiated state. Cell fusion and ACh receptors both represent aspects of the differentiation of the myoblast membrane. It is not yet known whether cyclic AMP influences also the cytoplasmic differentiation of muscle and therefore how completely it is coordinating the expression of the differentiated state.

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19. This work was supported by the Muscular Dystrophy Group of Great Britain.
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9 February 1981; revised 14 July 1981

## Epithelial Cell Volume Regulation: Bicarbonate Dependence

Abstract. *When* Necturus *gallbladder epithelial cells are osmotically shrunken, they rapidly return to their original volume despite the continued presence of a hypertonic bathing solution. This volume-regulatory process requires bicarbonate ions in the bathing solutions and is associated with the uptake of chloride ions. Volume-regulatory increase by epithelial cells is probably due to the parallel operation of sodium-hydrogen and chloride-bicarbonate exchangers in the apical cell membrane.*

Epithelial cells of *Necturus* gallbladder regulate their volume after a change in the osmolality of the solutions bathing either their apical or their basolateral surface (*1, 2*). When the osmolality of the solution bathing the apical (mucosal) surface is increased by the addition of mannitol, the cells shrink to a minimum size in about 40 seconds and then return to their original volume in about 90 seconds despite the continued hypertonicity of the apical bathing solution (Fig. 1, top). The rate of fluid flow into the *Necturus* gallbladder cell during volume-regulatory swelling is three to four times greater than the normal rate of fluid absorption by this tissue (*1–3*). Cell volume regulation after osmotically induced shrinkage probably involves the entry of NaCl from the mucosal medium into the cell across the apical cell membrane. Support for this conclusion comes from observations that volume-regulatory increase during perfusion of a hypertonic solution requires $Na^+$ in the mucosal bathing solution (*1–3*). The role of anions in volume regulation by epithelial cells has not been investigated.

We studied the $HCO_3^-$ dependence of volume regulation by *Necturus* gallbladder epithelial cells in response to hypertonic solutions on their apical cell surface. Gallbladders were mounted in a miniature Ussing chamber and visualized by a light microscope equipped with differential interference contrast optics; the cell volume was measured by planimetry of optical sections of the epithelial cells (*2–4*). When both perfusion solutions contained 10 m$M$ $HCO_3^-$, addition of 36-mOsm mannitol to the mucosal perfusion solution (an 18 percent increase in osmolality) caused typical cell shrinkage (14.4 percent) and subsequent volume-regulatory swelling (Fig. 1, top, and Table 1). Removal of all $HCO_3^-$ from the perfusion solutions did not affect the osmotically induced cell shrinkage (15.4 percent) but prevented the volume-regulatory swelling (Fig. 1, bottom, and Table 1).

We punctured the cells of *Necturus* gallbladder with voltage and ion-sensitive microelectrodes to study the alterations in apical membrane potential difference (PD) and intracellular $Cl^-$ activity ($a_{Cl}$) which accompany hypertonicity of the mucosal bath. The gallbladder was


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