Appearance of β-Adrenergic Receptors and Catecholamine-responsive Adenylate Cyclase Activity during Fusion of Avian Embryonic Muscle Cells*

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The appearance of β-adrenergic receptors and isoproterenol-stimulated adenylate cyclase during differentiation of embryonic quail muscle cells in tissue culture has been examined. β-Adrenergic receptors first appear during the fusion stage of myogenesis. [\( ^{125}I \)Iodohydroxybenzylpindolol (\( ^{125}I \)IYP)] was used as the ligand to characterize β-adrenergic receptors. In equilibrium binding studies, mature myotubes contained, per diploid nucleus, approximately 800 specific and saturable binding sites, which have a dissociation constant (K\(_d\)) for \( ^{125}I \)IYP of 108 pm. When determined from the association and dissociation rate constants, the kinetically derived \( K_d \) is 63 pm. \( ^{125}I \)IYP binding to myotube membranes is blocked stereospecifically by a number of β-adrenergic agonists and antagonists. In contrast, proliferating myoblasts contain fewer than 10 β-adrenergic receptors per cell.

Both myoblast and myotube membranes contain an adenylate cyclase activity which is stimulated by guanine nucleotides and fluoride, and is activated in membranes derived from cells treated with cholera toxin. Myotube membrane adenylate cyclase, but not myoblast membrane adenylate cyclase, is stimulated 2- to 3-fold by isoproterenol. Accumulation of cAMP by intact myotubes, but not by myoblasts, is stimulated 3- to 4-fold by isoproterenol. Both cell types accumulate cAMP in response to cholera toxin. These results indicated that as the embryonic muscle cells fuse, they develop specific β-adrenergic receptors that become functionally coupled to a pre-existing adenylate cyclase.

Each cell type generated during embryogenesis appears to develop a unique inventory of hormone receptors (1). The acquisition of responsiveness to different arrays of hormones during differentiation is probably critical for the normal development and survival of the organism. The biochemical processes involved in the development of hormone responsiveness by differentiating cells are readily studied in tissue culture.

Embryonic quail skeletal muscle culture offers several advantages as a model system for studying the development of hormonal responsiveness in a differentiating cell type. (i) The differentiation of these myogenic cells in tissue culture appears to be very similar to that observed in vivo (2, 3). (ii) Cultures of myogenic cells can be obtained which are not contaminated by other cell types; this is particularly important if the other cell types respond to the hormone. (iii) Myoblasts undergo a prolonged period of proliferation before differentiation if the cultures are initially plated at a very low cell density; therefore, early events in the differentiation process can be studied. (iv) Muscle cell fusion and differentiation occur in a very synchronous manner. These cultures often go from 0 to 70% fusion within 24 h. Additional muscle fusion then occurs over the next several days until greater than 90% of the myoblasts have fused. The multinucleate myotubes undergo further maturation; cross-striations and spontaneous contractions are observed 24 to 48 h after the fusion burst.

Epinephrine is known to influence the metabolism and contraction of skeletal muscle. In this report, we have examined the appearance of β-adrenergic hormone receptors as well as the development of hormone-stimulated adenylate cyclase during differentiation of embryonic quail skeletal muscle in tissue culture.

† Portions of this paper (including "Experimental Procedures," Figs. 1 to 3, 6, and 7, and Tables III and IV) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9560 Rockville Pike, Bethesda, Md. 20014. Request Document No. 79M-2083, cite authors, and include a check or money order for $1.50 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

RESULTS

Appearance of β-Adrenergic Receptors during Muscle Fusion—The data in the miniprint provide strong evidence that the \( ^{125}I \)IYP-binding sites on the particulate fractions isolated from well developed myotube cultures are authentic β-adrenergic receptors. We next determined when, during the differentiation of the muscle cells, β-adrenergic receptors appear. Specific binding of \( ^{125}I \)IYP to the particulate fraction from myotube cultures is readily apparent (Fig. 4A). However, no specific binding of \( ^{125}I \)IYP to an identical concentration of particulate fraction from proliferating myoblasts is detectable (Fig. 4B). We calculate that the \( ^{125}I \)IYP binding assay could detect 10 β-adrenergic receptors per cell.

This muscle tissue culture system has been adapted to give a prolonged proliferative phase followed by a rapid burst of muscle cell fusion and subsequent differentiation into well developed myotubes. Fig. 5 shows that β-adrenergic receptors appear following the burst of muscle cell fusion. There is about a 5-h lag period between muscle fusion and the appearance of β-adrenergic receptors.
Development of $\beta$-Adrenergic Receptors during Myogenesis

Lack of Effect of Catecholamines in the Medium on [\(^{125}\)I]IYP Binding to Myoblast Particulate—Chronic exposure of cells containing $\beta$ receptors to $\beta$ agonists reduces the number of receptors (20). The medium used for muscle culture includes embryo extract and horse serum, which may contain high concentrations of catecholamines. Therefore, the absence of $\beta$-adrenergic binding sites on the particulate fraction from proliferating myoblasts could have resulted from catecholamines in the medium. This possibility can be tested directly, since $\beta$ antagonists competitively block the ability of agonists to lower receptor number (21). Proliferating myoblast cultures grown for 48 h in fresh medium containing 10 $\mu$m sotalol, a powerful $\beta$ antagonist (Fig. 3B), did not result in the appearance of $\beta$-adrenergic binding sites (Table I). This concentration of sotalol, which is 500 times the $K_i$ for inhibition of [\(^{125}\)I]IYP binding to membranes (Fig. 3B), should prevent catecholamines in the medium from reducing the number of $\beta$-adrenergic binding sites on myoblast cultures. In addition, myoblast cultures incubated under the same conditions with sotalol exhibited no difference in specific [\(^{125}\)I]IYP binding compared to control myoblast cultures (Table I). Thus, extensive washing effectively removed the sotalol.

Stimulation of Adenylate Cyclase Activity in Muscle by $\beta$-Agonists—Evidence that myotubes, but not myoblasts, respond physiologically to $\beta$-adrenergic agonists is presented in Table II. After exposure to 10 $\mu$m isoproterenol, the cAMP level in myotubes is increased approximately 3-fold. However, the cAMP level in myoblasts does not increase after exposure to isoproterenol. The response of cells to $\beta$-adrenergic agonists may often be enhanced by preincubation of the cells with cholera toxin (22). CAMP accumulation during 10 min is enhanced in both myotubes and myoblasts by preincubation with cholera toxin (Fig. 5). A Time course of appearance of $\beta$-adrenergic receptors in differentiating muscle cultures. Particulate fractions of muscle cells cultured for the indicated times were prepared and assayed for [\(^{125}\)I]IYP binding as described under "Experimental Procedures." Values are the mean of quadruplicate determinations. Standard errors for myotube data (A) were less than 10%, and standard error bars for myoblast data (B) are omitted where errors were too small to draw conveniently. The difference between total (O) and nonspecific binding (C) represents specific binding.

**Table I**

<table>
<thead>
<tr>
<th>Culture</th>
<th>Additions</th>
<th>[(^{125})I]IYP binding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>cpm/sample</td>
</tr>
<tr>
<td>Myoblast</td>
<td>None</td>
<td>2029 ± 321</td>
</tr>
<tr>
<td>Myoblast</td>
<td>Sotalol</td>
<td>2243 ± 156</td>
</tr>
<tr>
<td>Myotube</td>
<td>None</td>
<td>4104 ± 351</td>
</tr>
<tr>
<td>Myotube</td>
<td>Sotalol</td>
<td>4221 ± 149</td>
</tr>
</tbody>
</table>

Not significant difference between plus and minus propranolol.

**Table II**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Myoblast culture</th>
<th>Myotube culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>No additions</td>
<td>3.1</td>
<td>9.0</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>2.8</td>
<td>30.3</td>
</tr>
<tr>
<td>Cholera toxin preincubation</td>
<td>11.9*</td>
<td>57.1</td>
</tr>
<tr>
<td>Cholera toxin preincubation plus isoproterenol</td>
<td>14.8*</td>
<td>82.7</td>
</tr>
</tbody>
</table>

*These values are not significantly different based on the variations in the assay.
for 1 h with 10 nM cholera toxin (Table II). However, the cAMP level in myotubes, but not myoblasts, is further increased by exposure to isoproterenol. Further properties are described in the miniprint.

**DISCUSSION**

Our results show that \( \beta \)-adrenergic receptors appear in developing embryonic skeletal muscle cells during the fusion stage of myogenesis. The cells also become responsive to isoproterenol following fusion. Well differentiated myotubes contain approximately 800 \( \beta \)-adrenergic receptors per diploid nucleus. We obtain approximately the same number whether we do the binding assay on the myotube particulate fraction or on intact cells on culture dishes (\( K_d = 142 \) pm, 1100 receptors per diploid nucleus). Simultaneous binding of \( \beta \) receptors have been reported in other cell types which respond to isoproterenol (14).

In these studies, we used \( [\text{^{125}}\text{I}] \text{HYP} \), which has proven to be a useful ligand for measuring \( \beta \)-adrenergic receptors in other systems (14, 25). Binding of \( [\text{^{125}}\text{I}] \text{HYP} \) to myotubes is to a single class of high affinity sites which demonstrate the characteristics of a \( \beta \)-adrenergic receptor (18). \( \beta \) receptors have been reported previously to be present in muscle (19).

In contrast, proliferating myoblasts lack detectable \( \beta \)-adrenergic receptors. The \( [\text{^{125}}\text{I}] \text{HYP} \) binding assay can detect as few as 10 receptors per cell and the binding assay is done on the total particulate fraction. Therefore, if myoblasts had \( \beta \) receptors on internal membranes, we would detect them in our binding assay. Current evidence suggests that plasma membrane proteins are synthesized on membrane-bound polysomes and remain membrane-bound during transit to the plasma membrane (26). Therefore, our results suggest that muscle \( \beta \) receptors are made de novo coincident with fusion.

We have attempted to eliminate some of the more trivial explanations for this phenomenon. Chronic exposure of cells to \( \beta \)-adrenergic agonists, but not antagonists, can decrease the number of \( \beta \)-adrenergic receptors (20, 21). Embryo extract and horse serum may contain high levels of catecholamines. However, no \( \beta \) receptors appear in proliferating myoblast cultures during a 48-h incubation in fresh medium containing large amounts of sotalol, a \( \beta \)-adrenergic antagonist. This concentration of sotalol (10 \( \mu \)M) would competitively block the ability of catecholamines in the medium to lower the receptor number.

Smilowitz and Fischback (27) have demonstrated that if embryonic chick pectoral muscle is dissociated without the use of proteolytic enzymes, then acetylcholine receptors can be detected on mononuclear myogenic cells. They suggest that previous studies did not detect acetylcholine receptors on these cells because proteolytic enzymes were used during tissue dissociation and cultured myogenic cells did not have time to recover lost receptors before fusion. We used low concentrations of trypsin to remove myoblasts from primary muscle cultures in order to establish pure secondary muscle cultures. Therefore, the apparent absence of \( \beta \)-adrenergic receptors on myoblasts may be the result of the trypsinization step. However, this explanation is very unlikely because our cultures are adapted to give a prolonged proliferative phase (8 to 9 generations) before fusion and should recover any lost \( \beta \) receptors.

Hormone binding to \( \beta \)-adrenergic receptors results in stimulation of adenylate cyclase. Measurements of the responsiveness of myoblasts and myotubes to \( \beta \)-adrenergic agonists correspond well with our \( \beta \) receptor binding results. Isoproterenol does not stimulate the rate of cAMP accumulation above basal levels in intact proliferating myoblasts and does not stimulate adenylate cyclase activity in myoblast homogenates. However, intact myotubes respond to isoproterenol with a 3- to 4-fold increase in cAMP accumulation above basal levels. Isoproterenol also induces a 2- to 3-fold increase in adenylate cyclase activity in myotube homogenates. Significant stimulation of adenylate cyclase activity in myotube homogenates occurs at 10 \( \mu \)M isoproterenol.

Adenylate cyclase activity in both myoblast and myotube homogenates shows the usual stimulation by guanine nucleotides and fluoride. In both cell types, the activity is also stimulated by preincubation with cholera toxin. The fluoride-stimulated adenylate cyclase specific activity remains relatively constant during the differentiation of muscle in tissue culture. These results suggest that adenylate cyclase and \( \beta \) receptors are under separate control during myogenesis. Other experimental approaches also have demonstrated that \( \beta \) receptors and adenylate cyclase are separate membrane components. These included: cell fusion between receptor- and cyclase-negative cells to form hormone-responsive hybrids (28); isolation of receptor- and cyclase-negative cell lines and in vitro reconstitution of catecholamine-sensitive adenylate cyclase activity (29); and, more recently, butyric acid was shown to induce \( \beta \)-adrenergic receptors in HeLa cells without increasing basal or fluoride-stimulated adenylate cyclase activity (30). Catecholamines appear very early in the embryonic development of birds (31, 32). Both epinephrine and noradrenaline have been detected in the chick embryo after 3 days of incubation. Therefore, the development of \( \beta \)-adrenergic receptors in bird muscle occurs long after the hormone is circulating in the embryo.

In several studies using rat muscle cell lines, it has been reported that unfused rat myogenic cells have \( \beta \)-adrenergic receptors (33-35) and respond to catecholamines with a stimulation of adenylate cyclase activity (34, 36, 37). Since our studies were done on avian embryonic muscle in tissue culture, we cannot exclude the possibility that the development of catecholamine responsiveness in muscle of other species differs from that in birds. However, results similar to ours have been reported by Mawatari et al. (38), who found that fusion human muscle cells (satellite cells) in culture do not respond to catecholamines with a stimulation of adenylate cyclase, whereas differentiated human myotube cultures do respond (38). Mawatari et al. (38) also reported that cultured muscle cells from patients with Duchenne's muscular dystrophy, unlike normal human muscle cells, do not respond to epinephrine with stimulation of adenylate cyclase. Hopefully, studies on the regulation of the \( \beta \)-adrenergic receptor and hormone-sensitive adenylate cyclase during the differentiation of skeletal muscle may provide insight into the nature of the lesion in this serious genetic disease.

**REFERENCES**

Development of β-Adrenergic Receptors during Myogenesis


SUPPLEMENTAL MATERIAL:

APPENDIX A: ABSOLUTE ADRENERGIC RECEPTORS AND CATECHOLAMINE RESPONSIVE ADENYLYL CYCLASE ACTIVITY IN CULTURED MUSCLE CELLS

E. J. DeMaria, J. E. Lightfoot, C. A. Bentley

EXPERIMENTAL PROCEDURES

Materials: [125]I] Iodination kit: Amersham International (UK) Ltd. [125]I] was used within 1 month purchased from New England Nuclear. [3H]Nal was used within 1 week of purchase. Various adrenergic agonists were obtained from several suppliers. (-)isoproterenol bitartrate; (-)propranolol bitartrate were from Sigma Chemical Company; (+)propranolol bitartrate; (-)isoproterenol bitartrate from Miles; (+)Nal bitartrate from Starting Point Laboratories; (-)isoproterenol bitartrate from Sigma. All other chemicals were obtained from commercial sources and were of the highest grade of purity obtainable.

Protocols: Cells were isolated from the portal of the mouse by differential density gradient (counted nucleated cells) and cultured in a medium of Eagle's minimal essential medium in Eagle's basal salt, supplemented with 0.1 M Hepes, 2 mM glutathione, 50 μM mercaptoethanol, 25 mM NaHCO3, 10 μM GTP, and 10 μM isobutylmethylxanthine. Isolated myocytes were counted. Mitochondria were isolated using a procedure for the isolation of mitochondria. Myocytes were injured using a procedure for the isolation of injured mitochondria. Mitochondria were injured using a procedure for the isolation of injured mitochondria.

Cyclic AMP Assay: Cells were incubated for 10 min in the presence of (-)isoproterenol or (-)propranolol. After incubation, the cells were harvested and the cyclic AMP content was determined by a procedure for the determination of cyclic AMP content.

RESULTS

The results of the cyclic AMP assay are shown in Figure 1. The cyclic AMP content of the control cells was found to be 2.2 nM. The cyclic AMP content of the cells incubated with (-)isoproterenol was found to be 2.2 nM. The cyclic AMP content of the cells incubated with (-)propranolol was found to be 2.2 nM. The cyclic AMP content of the cells incubated with (-)isoproterenol and (-)propranolol was found to be 2.2 nM. The cyclic AMP content of the cells incubated with (-)isoproterenol and (-)propranolol was found to be 2.2 nM. The cyclic AMP content of the cells incubated with (-)isoproterenol and (-)propranolol was found to be 2.2 nM. The cyclic AMP content of the cells incubated with (-)isoproterenol and (-)propranolol was found to be 2.2 nM. The cyclic AMP content of the cells incubated with (-)isoproterenol and (-)propranolol was found to be 2.2 nM. The cyclic AMP content of the cells incubated with (-)isoproterenol and (-)propranolol was found to be 2.2 nM. The cyclic AMP content of the cells incubated with (-)isoproterenol and (-)propranolol was found to be 2.2 nM. The cyclic AMP content of the cells incubated with (-)isoproterenol and (-)propranolol was found to be 2.2 nM. The cyclic AMP content of the cells incubated with (-)isoproterenol and (-)propranolol was found to be 2.2 nM. The cyclic AMP content of the cells incubated with (-)isoproterenol and (-)propranolol was found to be 2.2 nM.
The binding of $[^{3}H]$InHYP to myoblast particulate membranes is reversible in the presence of excess propylthiouracil and follows simple first-order decay for up to 120 min (Fig. 1B). The half-life of the ligand-receptor complex is 30 min and the reverse rate constant, $k_{-2}$, is 0.014 $\text{m}^{-1}\text{s}^{-1}$. From the kinetically determined values of $k_{+1}$ and $k_{-2}$, we calculate that the equilibrium dissociation constant is $1.6 \times 10^{-8} \text{M}$.

Dissociation of Concentration of Ligand. We obtained a separate estimate of the $K_{d}$ for $[^{3}H]$InHYP by measuring binding at equilibrium. We determined a functional ligand concentration. The non-specific binding component in the presence of 20 $\mu$M $[^{3}H]$InHYP appears to be a linear function of $[^{3}H]$InHYP concentration (Fig. 2A). Specific $[^{3}H]$InHYP binding appears to be a hyperbolic function and Scatchard analysis (Fig. 2) of the specific binding data from two experiments plus the evidence for the presence of two populations of binding sites with a $K_{d}$ of 100 $\mu$M. This calculated value for the dissociation constant is in fair agreement with the value (60 $\mu$M) calculated from kinetic data. Saturation of receptor sites was achieved within 15 min. Specific binding of $[^{3}H]$InHYP to particulate membranes from intact myotubes is saturated at $[^{3}H]$InHYP concentrations. This saturation of binding sites is normalized to DNA rather than protein, we calculate that intact myotubes have about 1000 specific binding sites per diploid myoblast nucleus.

Displacement of $[^{3}H]$InHYP by Beta-Adrenergic Agonists and Antagonists. $[^{3}H]$InHYP binding to myoblast particulate membranes is blocked by beta-adrenergic agonists and antagonists (Fig. 3). Binding of both agonists and antagonists to beta-adrenergic receptors has been characterized by stereospecificity. The (-) isomers being more potent ligands (19). Our results also indicate stereospecific inhibition of binding of $[^{3}H]$InHYP to crude particulate membranes of myotubes (Fig. 3). In addition, there is evidence of two separate populations of binding sites with approximate two orders of magnitude higher than the corresponding (-) isomers.

Inhibition of $[^{3}H]$InHYP binding to myoblast membranes by beta-adrenergic agonists and antagonists (Fig. 3). Binding of $[^{3}H]$InHYP to myoblast particulate fractions (100 $\mu$g protein/assay) was determined in the absence and presence of the indicated inhibitors as described in Experimental Procedures. Values have been corrected for non-specific binding as measured in the presence of 20 $\mu$M p-chloroamphetamine and are expressed as the percent inhibition of specific binding.

Beta-adrenergic receptors have been subclassified into beta, and beta, adrenergic receptors (18). In tissue characterized by beta, receptors, epinephrine is considerably more potent than isopropylamine in competing for radioligand binding. Beta-adrenergic receptors have been shown to mediate the metabolic effects of catecholamines in skeletal muscle (19). The relative potency of adrenergic agonists in binding to myoplasmic particulate fraction is in the order: isoproterenol, epinephrine > norepinephrine (Fig. 3). This potency series identifies these beta-adrenergic receptors as beta, receptors.

Properties of Adenylate Cyclase in Myotubes and Myoblasts. Myoblast homogenates have an adenylate cyclase activity with greater than 50% activity toward GTP. Pairwise comparison of adenylate cyclase activity in myoblast homogenates and myotubes have been shown to mediate the metabolic effects of catecholamines in skeletal muscle (19). In contrast, an adenylate cyclase activity with greater than 50% activity toward GTP is present in homogenates of myotubes (Table IV). At 10 $\mu$M (isoproterenol), the basal adenylate cyclase activity in myoblast homogenates is doubled and addition of 10 $\mu$M (isoproterenol) and 50 $\mu$M GTP doubles the adenylate cyclase activity compared to incubation with 10 $\mu$M GTP alone. Maximum activation occurs with 20 $\mu$M and half-maximal activation with 5 $\mu$M (isoproterenol) in the presence of 100 $\mu$M GTP (Fig. 4). Pretreatment of intact myotubes for 1 h with cholera toxin enhances the activation of adenylate cyclase by isoproterenol and by GTP.

Table III. Analytical Cyclase Activity in Myoblast Homogenates

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Adenylate Cyclase Activity (pmol/mg protein/min)</th>
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</thead>
<tbody>
<tr>
<td>No Additions</td>
<td>7.3</td>
</tr>
<tr>
<td>10 $\mu$M (isoproterenol)</td>
<td>6.6</td>
</tr>
<tr>
<td>Sodium Fluoride</td>
<td>42.5</td>
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<tr>
<td>GTP</td>
<td>74.3</td>
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<tr>
<td>OT plus 100 $\mu$M (isoproterenol)</td>
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<tr>
<td>GTP plus 10 $\mu$M (isoproterenol)</td>
<td>58.3</td>
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<tr>
<td>GTP plus 1 $\mu$M (isoproterenol)</td>
<td>56.6</td>
</tr>
</tbody>
</table>

Table IV. Analytical Cyclase Activity in Myotube Homogenates

Adenylate cyclase activity in myotube homogenates by isoproterenol. Influence of isoproterenol concentrations on adenylate cyclase activity in myotube homogenates (Table IV). Adenylate cyclase activity was determined as described in Experimental Procedures. Where indicated, 10 $\mu$M GTP and 10 $\mu$M (isoproterenol) were used.

Table V. Analytical Cyclase Activity in Myotube Homogenates

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Adenylate Cyclase Activity (pmol/mg protein/min)</th>
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</thead>
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<tr>
<td>No Additions</td>
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<tr>
<td>10 $\mu$M (isoproterenol)</td>
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<td>Sodium Fluoride</td>
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<td>GTP plus 1 $\mu$M (isoproterenol)</td>
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Table VI. Analytical Cyclase Activity in Myotube Homogenates

Adenylate cyclase activity in myoblast homogenates by isoproterenol. Influence of isoproterenol concentrations on adenylate cyclase activity in myoblast homogenates (Table VI). Adenylate cyclase activity was determined as described in Experimental Procedures. Where indicated, 10 $\mu$M GTP and 10 $\mu$M (isoproterenol) were used.