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Regulation of Adenylate Kinase and Creatine Kinase Activities in Myogenic Cells

(myogenesis/enzyme regulation)

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ABSTRACT **The regulation of the specific activities of adenylate kinase (EC 2.7.4.3) and creatine kinase (EC 2.7.3.2) in myogenic cell lines is independent of cell fusion. The observed increases in enzyme specific activities are cell density dependent, and may be further broken down into contributions from an increase in enzyme activity per cell and a decrease in protein per cell. Only the former appears to be affected by medium conditioning.**

There is an increase in the specific activities (enzyme activity per unit of total cellular protein) of adenylate kinase (EC 2.7.4.3; ATP:AMP phosphotransferase) and creatine kinase (EC 2.7.3.2; ATP:creatine *N*-phosphotransferase) temporally associated with cell fusion during myogenesis *in vivo* (1) and *in vitro* (2, 3). These post-fusion increases in enzymatic activities could either be dependent upon myotube formation, or not be functionally coupled with cell fusion. Since myogenic cells normally fuse to form multinucleate fibers, it has been difficult to experimentally dissociate the regulation of "differentiated" muscle metabolism and the morphological changes associated with myogenesis. In an attempt to answer the question of whether myotube formation is required for the expression of differentiated functions in myogenic cells, myotube formation has been inhibited *in vitro* by calcium deprivation (4) and by growth in 5-bromodeoxyuridine (BrdU) (2). These conditions also inhibited the normal increase in specific activities of creatine kinase and adenylate kinase. However, the possibility exists that both BrdU and calcium deprivation alter cellular functions other than those involved in the fusion process, resulting in a direct inhibition of the normal increase in adenylate kinase and creatine kinase activities.

Another approach to the question of enzyme regulation in myogenic cells is through the selection of nonfusing variants from clonal myogenic cell lines, and the use of these lines to separate the temporally associated events of cell fusion and increased adenylate kinase and creatine kinase activities. A nonfusing variant of Yaffe's L6 myogenic cell line (5) has recently been obtained (6). This paper describes experiments in which both L6 and its nonfusing variant were used to examine the regulation of adenylate kinase and creatine kinase in myogenic cells.

MATERIALS AND METHODS

The myogenic cell line L6 was derived from rat thigh muscle (3, 5, 7). A nonfusing variant of L6, called M3A, was obtained by selecting cells for their ability to grow in suspension culture (6). M3A grows in suspension in plastic petri dishes, but grows attached to the surface of tissue culture dishes. The cells do not fuse in either situation. M3A shows a hyperpolarizing response to iontophoretically applied acetylcholine comparable to that observed in L6 myoblasts (A. J. Harris, personal communication). The creatine kinase isozymes (8) of L6 and M3A are indistinguishable. All cells were cultured in modified Eagle's medium (9) containing 10% fetal-calf serum at 36°. Falcon plastic tissue culture or petri dishes were used as indicated. Cell number determinations and assays for creatine kinase and adenylate kinase were done as described (10). Cells were dissociated for cell number determinations and replating with 0.25% (w/v) Viokase (Gibco).

RESULTS

When L6 and M3A cells were plated at low cell densities, the activities of creatine kinase and adenylate kinase per unit of total cellular protein increased as a function of cell number in the cultures (Fig. 1). With L6 cells, the increase began during the exponential phase of growth and continued during myotube formation. However, M3A showed an increase in the specific activities of adenylate kinase and creatine kinase synchronous with L6, but in the absence of cell fusion. These increases in enzyme activities occurred regardless of whether M3A cells were cultured in suspension or attached.

A number of other clonal cell lines, including fibroblasts and hepatomas, were examined for similar changes during their growth cycles. The enzymatic activities of adenylate kinase and creatine kinase in these cells were less than one-tenth of those seen in L6 and M3A, and the specific activities increased less than 1.5-fold. There was a greater than 15-fold increase in specific activities of these enzymes between low-density exponentially dividing and stationary-phase M3A cells.

Since cell fusion is not requisite for the increased specific activities of adenylate kinase and creatine kinase in M3A and L6 cultures, a number of other control mechanisms were considered: (*1*) The enzyme specific activities could be a function of the number of divisions in the culture dish after plating. (*2*) Enzyme levels may be controlled by cell density. This could be dependent upon cell contact or coupled with medium conditioning (see below). (*3*) The specific activities may be regulated by medium conditioning, either through the addition or removal of a compound, or through the alteration of an existing medium component. A fourth possibility, that the increased specific activity is due to the cessation of cell division, is apparently ruled out by the data shown in Fig. 1, for the specific activities of creatine kinase and adenylate kinase increase in exponentially dividing cultures.

It is possible to rule out one of the first two possibilities in a single experiment. If the specific activities of adenylate kinase

Abbreviation: BrdU, 5-bromodeoxyuridine.

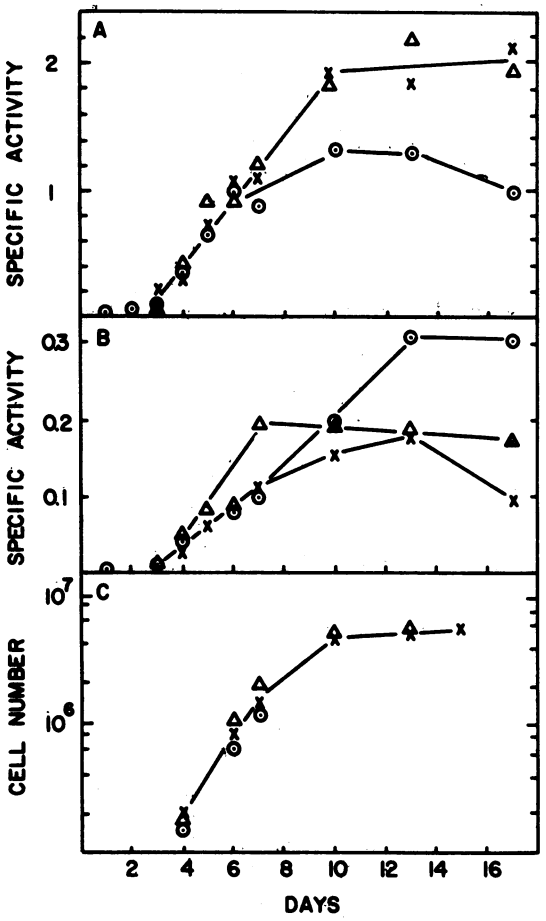

FIG. 1. Changes in the specific activities of creatine kinase and adenylate kinase in L6 and M3A cells as a function of their growth cycle. Cells were plated at 5×10^4 cells per 60-mm Falcon tissue culture or petri dish, and the cell number and specific activities of adenylate kinase and creatine kinase were followed as a function of the growth cycle. L6 cultures initiated cell fusion on day 7, making accurate cell counts impossible on the following days. Greater than 90% of the L6 nuclei were in fused myotubes on day 10. The enzymes' specific activities are expressed as ΔA_{412} per min per mg of protein. (*A*) Specific activity of adenylate kinase. (*B*) Specific activity of creatine kinase. (*C*) Viable cell number per 60-mm culture dish. (○) L6 grown on tissue culture dishes; (△) M3A grown on tissue culture dishes; (×) M3A grown in suspension culture on petri dishes.

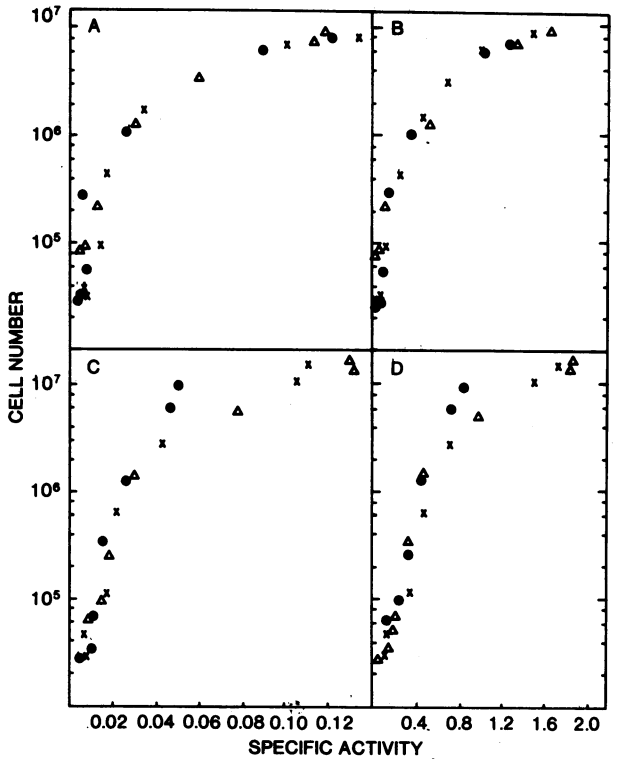

FIG. 2. Variation of enzyme specific activities as a function of cell density. Cells from exponentially growing M3A and L6 cultures were plated at initial densities between 2×10^4 and 1×10^7 cells per 60-mm tissue culture dish. Enzyme activities were determined on days 1, 2, and 3 after plating and expressed as a function of the cell number per dish at the time of assay. Activities are given as ΔA_{412} per min per mg of protein. (*A*) Specific activity of L6 creatine kinase. (*B*) Specific activity of L6 adenylate kinase. (*C*) Specific activity of M3A creatine kinase. (*D*) Specific activity of M3A adenylate kinase. (○) Day 1; (×) day 2; (△) day 3.

and creatine kinase are a function of cell density and not of division cycles, one would expect to see a change in specific activity before the onset of cell division in freshly plated cells. This new specific activity should reflect the cell density and be independent of division cycles. Thus, both L6 and M3A were harvested from exponentially growing cultures and plated at densities between 2×10^4 and 1×10^7 cells per 60-mm tissue culture dish. The cells were then assayed for creatine kinase and adenylate kinase on days 1, 2, and 3 after plating, and the specific activity of each enzyme was plotted as a function of the number of cells per dish at the time of assay (Fig. 2). There was no increase in cell number during the first 24 hr after plating, but there was an approximate doubling of cell number on each subsequent day for all but the two highest plating densities. At the highest plating density, L6 fused on day 3; the cell number used was that determined the previous day. The data in Fig. 2 show that the specific activities of the two enzymes change as a function of cell density and were independent of the number of division cycles, thus ruling out the first possibility.

The specific activities of adenylate kinase and creatine kinase have been defined as enzyme activity per unit of total protein in the cultures. However, when dealing with clonal cells, it is preferable to break down the components of the specific activity determinations into units of enzyme activity per cell and protein per cell. Thus, cells were plated over a wide range of densities and the relative contributions to enzyme specific activity by the above parameters were examined. The data in Table 1 show that changes in both protein per cell and enzyme activity per cell combine to generate the observed specific activities. The protein per cell decreased 4.5-fold and the enzyme activities per cell increased approximately 1.6-fold, together accounting for the observed 7-fold increase in specific activities. Since the determination of cell number and the total protein recovery were critical, the above experiment was repeated in three different ways to eliminate any possibility of an artifact in the experimental procedure. The cells were washed and lysed directly at 4°, or washed and scraped from the dishes with rubber policemen at room temperature, or removed from the dishes with Viokase and assayed for creatine kinase and adenylate kinase after neutralization of the proteolytic activity with serum and ex-

TABLE 1. *Enzyme activities as a function of cell density*

Cells per dish* × 10^4	mg of protein/ 10^6 Cells	Adenylate kinase		Creatine kinase	
		Specific activity	Activity/cell	Specific activity	Activity/cell
3.1	1.3	0.18	0.23	0.011	0.014
4.9	1.1	0.24	0.26	0.009	0.010
9.7	1.2	0.24	0.29	0.010	0.013
22	1.4	0.23	0.32	0.014	0.019
476	0.37	0.64	0.34	0.063	0.023
1100	0.29	1.3	0.36	0.068	0.020

Exponentially growing M3A cells were plated at the densities indicated, and the enzymatic activities, cell number, and protein concentrations were determined 2 days after plating. The cell number doubled after 2 days in all but the highest plating density, which remained constant. The specific activity is defined as ΔA_{412} per min per mg of protein, and enzyme activity per cell as ΔA_{412} per min per 10^6 cells.
* Values should be multiplied by 10^4.

tensive washing. The three procedures gave indistinguishable results with respect to all of the parameters shown in Table 1. Similar results were also obtained if Viokase was not used to separate cells before plating.

Both enzyme activity per cell and protein per cell change during the growth of M3A cells. The changes could result from intercellular contacts or from medium conditioning. If cells condition medium at a constant rate per cell, this conditioning may be a function of cell density and, thus, may be confused with other types of density-dependent phenomena, such as cell contact. To distinguish between these alternatives, M3A cells from low exponential growth were plated at low density in conditioned medium from stationary-phase cells. As controls, these cells were plated at the same density in fresh medium and also at high density in fresh medium. Protein per cell, enzyme per cell, and the specific activities of adenylate kinase and creatine kinase were determined on days 2 and 3 after plating. The data in Table 2 show that growth-conditioned medium did not alter the amount of protein per cell, but did affect the enzyme activities per cell and, thus, the specific activities of adenylate kinase and creatine kinase. Similar experiments have shown that conditioned medium can induce up to a doubling of enzyme activities per cell over control cultures within 24 hr, without affecting the protein per cell.

DISCUSSION

The following conclusions may be made on the basis of the above data: (*1*) The increase in the specific activities of adenylate kinase and creatine kinase normally temporally associated with myogenesis is not dependent upon cell fusion (Fig. 1). (*2*) The increase in the specific activities of these two enzymes depends on cell density (Fig. 2), and the specific activity changes can be broken down into two components: (*a*) a decrease in protein per cell that depends upon cell density (Table 1) and (*b*) an increase in enzyme activity per cell that is mediated by growth-conditioned medium (Table 2).

The inhibition of myoblast fusion in primary cultures by calcium deprivation or BrdU blocks the increase in creatine kinase and adenylate kinase activity temporally associated with cell fusion (2, 4). As stated previously, these negative results could be the result of detrimental effects on cellular metabolism and not reflect a true causal relationship between cell fusion and enzyme activity increases. In support of this possibility is the observation with primary chick-myoblast cultures that the inhibition of fusion by calcium deprivation does not block the appearance of the acetylcholine receptor (11). The appearance of this receptor is normally associated with fused myotubes both in primary (12, 13) and clonal myoblast (14) cultures.

It should also be noted that properties associated with differentiated muscle have been observed, albeit very infrequently, in mononucleate cells of primary cultures (15). However, the origin of this type of cell in primary cultures has been disputed, for it is possible that some mononucleate cells in these cultures are derived from muscle fibers fused *in vivo*. This makes it difficult to generalize from data on single cells. The situation is unambiguous with the myogenic line L6

TABLE 2. *Effect of conditioned medium on enzyme activities*

	Day 2			Day 3		
	Control	Conditioned medium	High cell density	Control	Conditioned medium	High cell density
Relative cell number	2.4	1.15	1.27	7	1.40	1.28
Protein per cell	1.24	1.36	0.307	1.19	1.29	0.323
Adenylate kinase (specific activity)	0.203	0.331	1.44	0.270	0.366	1.62
Adenylate kinase (activity per cell)	0.253	0.449	0.439	0.295	0.471	0.523
Creatine kinase (specific activity)	0.009	0.021	0.111	0.014	0.019	0.097
Creatine kinase (activity per cell)	0.011	0.029	0.034	0.015	0.025	0.032

Exponentially growing cells were plated at 2×10^5 cells per dish in conditioned medium from early stationary-phase cells or in fresh medium. In both cases the medium was changed daily. As a positive control, cells were also plated at 1.2×10^7 cells per dish in fresh medium. The cell number relative to day 1 after plating, enzyme activities, and protein per 10^6 cells were determined and expressed as described in Table 1.

and its M3A derivative, both of which show a density-dependent regulation of creatine kinase and adenylate kinase activities that is independent of cell division. This suggests that for these two enzymes, which are normally associated with the more differentiated state of muscle, there is no qualitative change during myogenesis, but only a quantitative increase in enzyme specific activity as a function of cell density. Since the adenylate kinase and creatine kinase activity is low at low cell densities, it may have been missed in primary cultures and in L6, thus making the increase appear qualitative. Thus, before it can be concluded that there is a qualitative enzymatic change associated with cell fusion, it is necessary to follow the enzyme activity throughout the growth curve of the cells from low plating densities. This has not been previously done (see, for example, refs. 3 and 10).

Data have been presented that suggest that conditioned medium has an effect on myotube formation in primary cultures (16, 17), and that the rate of cell fusion is dependent on cell density (16, 18). Although creatine kinase and adenylate kinase activities were not assayed in those studies, the apparent enhancement of myogenesis by increased cell density and conditioned medium is in agreement with the data presented above.

Both bacteria (19) and fibroblasts (20) are subject to a protein loss in the stationary phase of growth. Other cell lines acquire more protein per cell or increase in cell size as they progress through their growth curve (21, 22). By using volume determinations from a Coulter Counter, approximately 75% of the protein loss in M3A can be accounted for by a decrease in cell volume (unpublished observation). This suggests that there is either enhanced turnover of the non-creatine kinase and adenylate kinase proteins, or a selective loss of protein from the cells.

Since evidence has been presented for the density-dependent differentiation of several tissues *in vivo* (23), clonal cell cultures may be useful for the delineation of these and similar processes that are too difficult to approach experimentally *in vivo*.

It is sometimes assumed that the most differentiated end cells possess a set of properties that make them qualitatively different from their immediate developmental precursors and from other classes of terminally differentiated cells. Thus, unless the fertilized egg possesses small quantities of the macromolecules required for the expression of all differentiated functions, there is a qualitative (all or nothing) imposition of various properties characteristic of the maximally differentiated cell at one or more stages during embryogenesis. The question is, when during development do these changes occur? The evidence presented above and that obtained from the study of other classes of mesodermally derived cells suggest that this phenotypic change may take place before the primordium common to all cells of mesodermal origin. A comparison of fibroblasts and muscle is instructive in this regard. Although at first sight these types of cells and their immediate developmental precursors appear to be markedly different, a closer examination shows that they share a number of traits. (*1*) The immediate developmental precursors to differentiated skeletal (24) and smooth (25) muscle cells are electrically excitable; evidence has been presented that suggests that fibroblasts are capable of giving an active electrical response (26). (*2*) Skeletal muscle myoblasts (14), fibroblasts (27), and some smooth muscles (28) respond to acetylcholine with a slow hyperpolarization. (*3*) Fibroblasts (22, 29), smooth muscle (Tarikas and Schubert, unpublished observation), and skeletal muscle (29) all contain detectable amounts of true acetylcholine esterase. (*4*) Fibroblasts (30), skeletal muscle (10), and smooth muscle (25, 31) synthesize collagen. (*5*) The immediate developmental precursors to skeletal muscle myotubes synthesize myosin heavy chain, although at a slower rate than in the more differentiated cells (10). Myosin has also been found associated with fibroblasts (32) and, of course, smooth muscle. Taken together, these data indicate that a number of properties normally associated with three differentiated end cells of mesodermal origin are shared among the three cell types. Some of these traits are then amplified relative to others in order to generate the quantitative differences observed among the different end cells. In addition, when studied with sufficiently sensitive methods, the immediate developmental precursors to these end cells express most of the functions normally associated with the most differentiated cell in the sequence, but in a quantitatively reduced amount. For example, mononucleate skeletal muscle myoblasts are weakly excitable relative to myotubes (24), contain low levels of adenylate kinase and creatine kinase (Figs. 1 and 2), synthesize myosin heavy chain at 15% the maximum rate observed in myotubes, and show only quantitative differences in protein secretion during myogenesis (10). Since different terminally differentiated mesodermal cells and their immediate precursors share a common set of properties [which are apparently distinct from those of cells of other embryological origins, e.g., plasmacytes do not secrete collagen (10)], it follows that if any qualitative change occurred with respect to the synthesis of these "specialized" molecules, it had to take place prior to the genesis of primordial mesoderm. As the sensitivity of our assays for "differentiated" functions increases, it is likely that more similarities will be found among various cell types of common embryological origins.

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