CHANGES IN ADENYL CYCLASE SPECIFIC ACTIVITY DURING DIFFERENTIATION OF AN ESTABLISHED MYOGENIC CELL LINE.

J.P. Wahrmann, D. Luzzati and R. Winand.

Insitut de Biologie Moléculaire - Faculté des Sciences
Université de Paris VII - 2, Place Jussieu - Paris 5° - France

Received April 3,1973

Summary : The variation of specific activity of adenyl cyclase has been studied during differentiation of an established line of myoblast, strain L_6D and of a temperature sensitive developmental variant strain, H_6, derived from it. The specific activities of both basal and NaF stimulated adenyl cyclase were found to decrease 2 to 3 folds after fusion of myoblasts into myotubes in cultures of L_6D. Cultures of strain H_6 displayed the same decrease in specific activity of adenyl cyclase when grown at temperature which allows differentiation, while no decrease was observed at the temperature which does not allow cell fusion. These results indicate that the decrease in specific activity of adenyl cyclase is associated with cell fusion and reflects membrane changes ocurring during differentiation of myogenic cells.

Myoblast cultures of line L_6 (1) exibit many of the features characteristic of "in vivo" muscle differentiation. The mononucleated myoblasts grow exponentially until a confluent monolayer is established (6^{th} to 7^{th} day of culture), and then start to fuse into multinucleated myotubes. The proportion of cells which have fused into myotubes increases during the next 3 to 4 days, while characteristic muscle proteins accumulate,in close correlation with the fusion process (2) (3).

Changes in cell surface are certainly involved in fusion, and myoblast plasma membrane probably differs from those of the myotubes in many respects. As adenyl cyclase is an enzyme attached to the cell membrane, it could be taken as a witness of changes in membrane properties during muscle cell differentiation. We have consequently studied the specific activity of adenyl cyclase in cell extracts and purified membranes from myoblast cultures of line L_6 at different stages of differentiation and in those of a developmental temperature sensitive variant derived from this line (3).

Present adress : Institut de Mèdecine - Hôpital de Bavière
4000 - Liège - Belgium

MATERIAL AND METHODS

Myoblast cultures of line L_6D, a subclone of line L_6, isolated by Yaffé from rat skeletal muscle (1), were grown in 60 mm Falcon tissue culture dishes, in Waymouth medium (Eurobio Way 011) containing 0,5% embryo extract and 10% horse serum in a 5-7% CO_2 atmosphere at 37°. Cultures were usually seedeed at 2 x 10^4 cells/plate. Under these conditions myoblasts grow exponentially until a confluent monolayer is established (5^{th} to 6^{th} day of culture). During subsequent incubation (7^{th} to 12^{th} day of culture) an increasing proportion of cells form myotubes : by the end of the fusion period 30 to 80% of the cells present on the plates have fused into myotubes. A thermosensitive variant of this line, H_6, was used in some experiments. This variant was isolated after mutagenic treatment of line L_6 (3) : its growth is identical to the parent strain both at 37° and 40°, however it does not fuse into myotubes at 37°, while fusing normally at 40°.

Adenyl cyclase was measured by the method of Krishna et al. (4) with the addition of an ATP regenerating system. Unless stated otherweise, myoblasts cell extracts were incubated in the presence of 5 mM ATP, 22 µCi of ^{32}P - αATP (1100 mCi/nmole), 6, 2 mM $MgCl_2$, 42 mM Tris HCl pH 7,7, 16 mM teophylline, 0,2% albumin, 3,2 mM phosphoenol pyruvate and 100 µg/ml pyruvate kinase. Incubation was carried on for 10 minutes at 37°C and stopped by the addition of 0,1 ml of a solution containing a large excess of ^{32}P-ATP and ^{3}H-cyclic AMP (5). The ^{32}P-cyclic AMP formed during the reaction was separated by passage over a column of Dowex 50 and two subsequent precipitations by $ZnSO_4$ 8,5.10^{-3}M and $Ba(OH)_2$ 7,5.10^{-3}M. Protein concentration was determined by the method of Lowry et al. (6), using cristalline serum albumin as standard.

Myoblast plasma membrane were prepared by the method of Neville (7) as modified by Winand and Luzzati (in preparation).

RESULTS AND DISCUSSION

The specific activity of adenyl cyclase was measured in myoblast cultures at different stages of differentiation. Enzymatic reaction was linear for protein concentrations which varied over a range of 250 to 500 µg for non differentiated cells, and from 250 to 1000 µg for differentiated cells ; at higher protein concentrations, an inhibition of adenyl cyclase activity was observed.

Table I

Changes in the specific activity of adenyl cyclase during differentiation of myoblasts from the line L_6D

Stage of the culture	Assays made on	cAMP (pmoles/min./mg.protein)
Exponentially growing myoblasts (5th day of incubation)	crude homogenate	500
Differentiated cultures (12th day of incubation)	" "	200
Exponentially growing myoblasts (5th day of incubation)	purified membranes	540
Differentiated cultures (12th day of incubation)	" "	240

The value for crude homogenates represent the average of 8 separate experiments and those for purified membranes the average of 3 separate experiments, each involving duplicate determinations. All cultures were grown at 37°C.

Table II

Changes in specific activity of adenyl cyclase in cell extracts from cultures of the thermosensitive variant H_6

Stage of the culture	Temperature	cAMP (pmoles/min./mg.protein)
Exponentially growing myoblasts (5th day of incubation)	40°C	300
Differentiated cultures (12th day of incubation)		144
Exponentially growing myoblasts (5th day of incubation)	37°C	290
Stationary phase (15th day of incubation)		752

At permissive temperature (40°C) cell fusion begins on the 7^{th} day of incubation as with the parent strain. At non permissive temperature (37°C) the stationary phase of growth begins on day 7^{th} and continued up to the 15^{th} day of incubation. The values given represent the average of 3 separate experiments, each involving duplicate determinations.

Before fusion, no significant difference in adenyl cyclase activity could be detected between extracts from exponenentially growing cells and those from myoblasts having just attained the stationary phase of growth. As soon as cells began to fuse (0,04 myotubes/unit area) a decrease of 20% in enzyme specific activity could be observed, attaining 65 to 75% when the fusion process reached completion. On an average, the specific activity of adenyl cyclase was to two three times lower in homogenates from differentiated cells than in those from non differentiated myoblasts. The average value of eigh different experiments is given in table 1 (crude homogenate). Despite of variations in the level of adenyl cyclase activity between measurments, this decrease in specific activity of the enzyme was noted in each individual experiment.

The sensitivity of adenyl cyclase to fluoride activation was assayed in extracts from differentiated and non-differentiated cells. As it can be seen from figure 1, the activation of the enzyme by NaF increased over a concentration range of 2.10^{-3}M to 10^{-2}M. Both differentiated and non-differentiated cell extracts displayed a five fold increase in adenyl cyclase specific activity over the basal level at optimal NaF concentration. A twofold difference in adenyl cyclase activity between non-differentiated and differentiated cells extracts persited however after NaF activation. (fig.1).

However, the protein concentration per plate increases two to three fold during the fusion process, at a time when contractile proteins and many enzymes characteristic of the differentiated state accumulate. In order to investigate whether the observed decrease in specific activity of adenyl cyclase in differentiated cells was real or merely due to an increase in the protein content of the cells, relative to membrane proteins, we tested adenyl cyclase in purified membranes. As it can be seen from table 1, the specific activity of adenyl cyclase was 2 to 3 times lower in membrane isolated from differentiated cells than in those obtained from non-differentiated cells. It can be conclude that the differences in adenyl cyclase activity observed in crude homogenates is not due to the accumulation of intracellular proteins by differentiated myotubes, but reflects a difference in activity or quantity of the membrane bound enzyme.

To ascertain the relationship between differentiation and diminution in the specific activity of adenyl cyclase, we assayed the activity of the enzyme in cells extracts of a temperature sensitive developmental variant of line L_6, H_6 (3), which fuses into myotubes at 40°C (permissive temperature) but not at 37°C (non-permissive

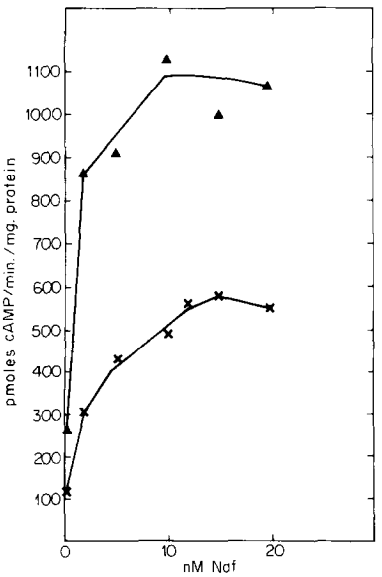

Fig. 1 Activation of adenyl cyclase by NaF in crude homogenates from (▲) non differentiated and (×) differentiated cells. Each experimental point represents the average of a duplicate determination. Non differentiated cells correspond to exponentially growing cultures 3 to 5 days incubation; differentiated cells are to fully fused cultures (9^{th} to 12^{th} day of incubation under conditions described in Materials and Methods).

temperature). At the permissive temperature we observed the same phenomenon as in cells of line L_6 (table 2) i.e. the basal level of adenyl cyclase specific activity was 3 times higher in exponentially growing non-differentiated cells, than in differentiated cells. Furthermore cultures of H_6 which were incubated at the non-permissive temperature (37°) displayed a 2 to 3 fold increase in the specific activity of adenyl cyclase between the time the cells reached confluency (7^{th} day) and the stationary phase of growth (15^{th} day of incubation). This feature does not seem to be particular to strain H_6, for the activity of adenyl cyclase, either basal or NaF activated, has been found to increase in many non differentiating cell types, when the cultures become confluent and enter the stationary phase of growth (8).

Thus, H_6 displays an increase in adenyl cyclase activity under conditions where it is unable to differentiate after reaching confluency, whereas there is an increase under conditions which allow fusion. We may conclude from the preceeding results that the decrease of adenyl cyclase activity observed with strain L_6D which occurs after

fusion is closely related to the fusion process and reflects changes in cell membrane properties associated with myoblast differentiation.

ACKNOWLEDGMENTS

We thank Professor François Gros for encouragement and stimulating discussions. This work was supported by funds of the Centre National de la Recherche Scientifique, the Fondation pour la Recherche Médicale Française and the Commissariat à l'Energie Atomique.

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