THE EFFECT OF A TRANSIENT INCREASE IN INTRACELLULAR CYCLIC AMP UPON MUSCLE CELL FUSION

Rosalind J. ZALIN and Ronald LEAVER
School of Biological Sciences, University of Sussex, Falmer, Brighton, Sussex, England

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1. Introduction

Increasing the intracellular level of cyclic AMP by the addition of dibutyryl cyclic AMP has been shown to promote differentiated function in a number of cell types. For example dibutyryl cyclic AMP stimulates axon formation in neuroblastomas, promotes collagen synthesis in chinese hamster ovary cells [2] and both the synthesis and secretion of sulphated acid polysaccharides in transformed fibroblasts [3]. Dibutyryl cyclic AMP has also been shown to enhance the differentiation of embryonic lens epithelial cells, promoting both the growth phase necessary for overt differentiation and the appearance of the γ-crystallins [4].

The effects of dibutyryl cyclic AMP upon the muscle differentiation, however, have been variable. Its addition 24 hr after plating to either a primary culture of chick myoblasts [5] or a rat muscle cell line [6] inhibited the onset of cell fusion in the cultures, whereas when added to a tumour cell line (16A) at the start of culture, the cyclic nucleotide had the converse effect of promoting the differentiation process [7]. Cell fusion is one of the initial events in the expression of the differentiated state of muscle cells and its sensitivity to raised levels of cyclic AMP suggests that the cyclic nucleotide may be involved in the control of expression of this aspect of muscle differentiation.

More recently we have examined the intracellular levels of cyclic AMP normally present in differentiating chick myoblasts and have found a 10-fold transient increase in cyclic AMP just 5 to 6 hr before the onset of cell fusion in the cultures [8]. The close correlation between the increase of cyclic AMP in the cells and the onset of myoblast fusion suggests that the transient increase in the cyclic nucleotide might be responsible for the overt expression of the muscle cell's differentiated state.

In an effort to reproduce the spontaneously occurring transient increase in cyclic AMP and to test the possibility that it is this increase in cyclic AMP which is the signal for cell fusion, experiments were carried out in which prostaglandin E_1 was added to the undifferentiated chick myoblasts. Prostaglandin E_1 was chosen because of its ability to produce both large and transient increases in intracellular cyclic AMP [9].

2. Materials and methods

2.1. *Skeletal muscle cell cultures*

These were prepared from 11–12 day embryonic chick thigh muscle using a method described previously [5]. The cells were seeded at a density of 2×10^6, grown in 85 mm Falcon tissue culture dishes coated with rat tail collagen and placed in a humidified 37°C incubator in an atmosphere of 95% air + 5% CO_2. The two types of medium used (Ham's F10 and Medium 199) were supplemented with 10% horse serum (Bio Cult. Ltd.) 2% chick embryo extract, glutamine (1mM), penicillin (50 units/ml) and streptomycin (50 μg/ml) and the pH of the complete medium finally adjusted by the addition of 10% $NaHCO_3$. The myoblast culture medium was changed only once at 24 hr after plating, when Ham's F10 was replaced with Medium 199 and care taken to keep culture conditions constant for all the experiments described. Where appropriate 50 μl of PGE_1 in 96% ethanol was added to each plate to give a final concentration of PGE_1 of 5 μg/ml. An equal vol

of ethanol was also added to the control cultures and all the additions were made at 37°C to minimise any effects due to temperature change.

2.2. *Measurement of cell fusion*

Cell fusion was estimated by direct microscopic examinations of methanol fixed and Ehrlich acid haematoxylin stained cultures. Using an eyepiece grid and at a magnification of 250, counts were made of the nuclei in single cells and in multinucleate units in 16 separate fields spaced evenly over the culture dish. Total counts/dish of between 500 and 2000 nuclei were obtained. Nuclei were put in the category of multinucleates only if three or more were clearly within the same cell membrane.

2.3. *Adenosine 3' : 5' - cyclic monophosphate assay*

A saturation assay method described by Brown et al. [10] was used to measure cyclic AMP concentrations in cell homogenates acidified with 5% trichloroacetic acid. 1 ml of 5% trichloroacetic acid was added directly to the muscle cultures after rapid decanting of the culture medium and the samples prepared for cyclic AMP estimation as described previously [8]. 95% of the cyclic AMP measured using this assay was lost after treatment with phosphodiesterase.

2.4. *DNA estimation*

The DNA content of the cultures was measured using a modification of the method of Kissane and Robins [11]. The DNA was extracted from the precipitate obtained after trichloroacetic acid addition to the muscle cultures by the addition of a further ml of 5% trichloroacetic acid and heating at 80% for 30 min. 0.5–4.0 μg of calf thymus DNA (Sigma) in 5% trichloroacetic acid was used to produce a linear standard curve.

3. Results and conclusions

Under the conditions of culture adopted in which the medium is renewed once after 24 hr of culture most of the myoblasts pass through at least one round of DNA synthesis and cell division before cessation of proliferation and cell division. The main burst of cell fusion begins at approx. 44 hr of culture, 5–6 hr after the observed increase in cyclic AMP [8]. Prostaglandin

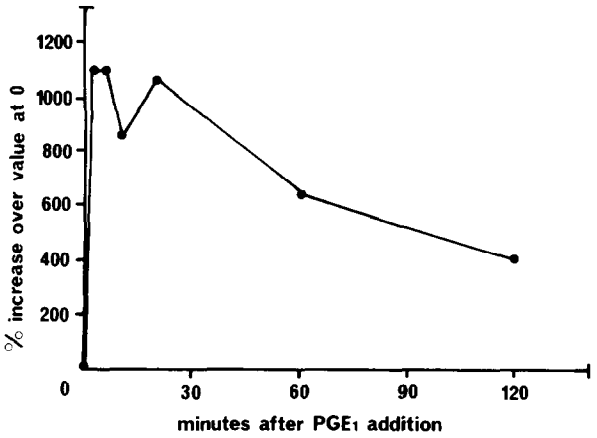

Fig.1. Myoblast intracellular cyclic AMP levels after the addition of PGE_1 at 34 hr of culture. Abscissa: time after PGE_1 addition in minutes. Ordinate: percentage increase in cyclic AMP above the value at O. Each point represents the mean value obtained from 3 experiments.

E_1 at a final concentration of 5 μg/ml was added to the plates during the prefusion phase of culture at 34 hr. Cells were then prepared for cyclic AMP estimations over the subsequent 2 hr period by the direct addition of trichloroacetic acid to the culture dishes (after removal of culture medium) and elution of the acid soluble material from Dowex 50 columns as described in Materials and methods. The intracellular cyclic AMP level was found to rise sharply after prostaglandin addition (fig.1), a maximum value 1100% above that of the basal level being obtained 2 min. after prostaglandin E_1 addition, at the first time of assay. The intracellular cyclic AMP level then fell gradually over the remaining period examined, yielding a level approx. 400% above that of the basal after 2 hr. The initial cyclic AMP increase obtained after PGE_1 addition is of a similar magnitude to that occurring spontaneously in the chick myoblast 5–6 hr before the onset of cell fusion in the cultures and in both cases the large increase is sustained for only a short time.

The ability of the prostaglandin to produce such a cyclic AMP increase was then made use of in an attempt to obtain more information about the relationship between the naturally occurring increase in cyclic AMP and the differentiation process. In particular, we wished to test the suggestion made above that it is the transient increase in intracellular cyclic AMP which is

acting as a signal for the onset of myoblast cell fusion. With this end in view, the time course of fusion was examined after the addition of prostaglandin E_1 to the myoblasts at 34 hr of culture. Since the cyclic AMP increase under control conditions occurs between 38–39 hr of culture, the prostaglandin is generating a cyclic AMP peak approx. 4–5 hr earlier than normal. Plates were taken at 2 hr intervals and cell fusion assessed by counting the nuclei in single cells and myotubes in a total of 16 separate fields. The fusion curves obtained (fig.2) suggest that the prostaglandin is bringing forward both the onset of cell fusion and the increase of nuclei into myotubes. The fact that the whole fusion process is altered in this way suggests strongly that the prostaglandin is affecting a substantial number of cells.

Increasing the intracellular level of cyclic AMP has been shown to affect the growth-rate of a number of cell types [12–14] and the possibility existed that the apparent increase in cell fusion was not due to a direct effect of prostaglandin E_1 upon the differentiating myoblasts but rather the result of a reduced growth of the non-fusing cell population. Such an explanation for the differences observed in the fusion time course after the prostaglandin-induced peak of cyclic AMP, however, is unlikely in view of the absolute increases obtained for the number of cells in myotubes in the prostaglandin treated cultures (table 1) and the absence of any consistent differences in the total numbers of cells present under the two sets of conditions. Thus,

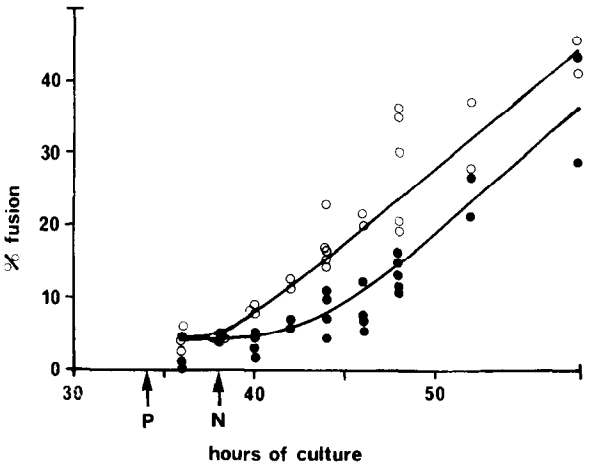

Fig.2. The effect of PGE_1 upon myoblast fusion. Abscissa: time of culture in hours; Ordinate: percentage fusion. Myoblast fusion, under control conditions (●---●) and after the addition of PGE_1 at 34 hr of culture (○---○). Appearance of cyclic AMP peak under normal conditions ↑N; + PGE_1 ↑P.

the results are consistent with the view that prostaglandin E_1 is affecting the fusion process directly, an explanation which is strengthened by the finding that the gap between the time courses of fusion under the prostaglandin treated and control conditions is 4–5 hr, exactly the same time interval as that between the artificially produced cyclic AMP increase and the peak which normally occurs in the cultures 5–6 hr before

Table 1
Total numbers of nuclei in single myoblasts and in myotubes under control conditions of culture and in the presence of prostaglandin E_1

Time of culture	Number of fused cells	Total number of cells in 16 fields
Control 36 hr	11.3 ± 3.9	479.5 ± 49.4
+ PGE_1 36 hr	20.6 ± 3.1	464.7 ± 31.4
Control 40 hr	25.0 ± 3.6	621.8 ± 64.7
+ PGE_1 40 hr	62.8 ± 10.7	547.0 ± 38.1
Control 44 hr	69.6 ± 10.9	830.2 ± 27.6
+ PGE_1 44 hr	102.6 ± 26.0	554.0 ± 95.7
Control 48 hr	124.8 ± 14.3	922 ± 62.8
+ PGE_1 48 hr	314.8 ± 45.3	1003.8 ± 62.2

Mean values obtained from a minimum of 5 experiments ± S.E.

fusion. This also means that the time interval between the cyclic AMP peak and the onset of fusion is maintained and provides direct evidence that the cyclic AMP increase is acting as the signal for cell fusion.

A possible explanation of the conflicting effects that dibutyryl cyclic AMP has been found to have upon muscle cell fusion [5–7] may lie in the fact that a raised intracellular cyclic AMP level of much longer duration than that normally present in the cells is likely to occur under such conditions. Depending upon its time of addition the high cyclic AMP level produced may either obscure or distort the naturally occurring transient cyclic AMP peak. The increase in intracellular cyclic AMP level which occurs as a result of hormonal stimulation of adenylate cyclase is often transient but so also is the hormone's effect upon the cell. In the differentiating muscle cell it would seem that the transient increase in the cyclic nucleotide in giving rise to cell fusion is producing an irreversible change in the muscle cell. Finally, the finding that cell fusion is enhanced by the addition of an agent known to increase cyclic AMP by stimulation of the cells' adenylate cyclase suggests that a similar interaction between the myoblast adenylate cyclase and an external factor may be responsible for the spontaneously occurring cyclic AMP peak. An explanation of this kind which implicates an external factor in the control of the expression of the differentiated state is compatible with the large amount of evidence which exists for a critical interaction between the muscle cells and the culture medium in myoblast differentiation [15,16].

It should be emphasized that this communication presents evidence of a positive controlling function for cyclic AMP only with respect to cell fusion and information concerning its effects upon other parameters of muscle differentiation is required to establish how general a signal cyclic AMP is in the expression of the muscle cells differentiated state.

Acknowledgement

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