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PROTEIN KINASE AND CYCLIC AMP LEVELS IN DIFFERENTIATING MYOBLASTS ARE ALTERED BY EXTRACELLULAR CALCIUM

G. E. Morris and Nguyen thi Mân

School of Biological Sciences, University of Sussex,
Falmer, BRIGHTON, BN1 9QG, U.K.

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SUMMARY

Protein kinase specific activities and cyclic AMP levels show a similar pattern of response, when the Ca^{2+} concentration is altered in the culture medium of differentiating chick skeletal muscle cells; an increase at intermediate Ca^{2+} concentrations (0.05-0.2mM), followed by a decrease at higher concentrations (2mM). Effects of Ca^{2+} on protein kinase appear to be on cyclic AMP-independent enzymes in both nucleus and cytoplasm, and are quite the reverse of Ca^{2+} effects on the muscle-specific enzyme, creatine kinase.

INTRODUCTION

Addition of different amounts of Ca^{2+} to Ca^{2+}-depleted medium results in an inhibition at intermediate concentrations (0.05-0.2mM), but not at higher concentrations (2-3mM), of muscle-specific creatine kinase (MM-CPK) accumulation by differentiating myoblasts (1). The mechanism of these Ca^{2+} effects is unknown, but one possibility is that cyclic AMP and/or cytoplasmic protein kinases act as intermediates, since interrelationships between Ca^{2+} and cyclic AMP levels are known to exist (2) and some protein kinases are Ca^{2+}-dependent (3).

The object of the present study was to determine the response to external Ca^{2+} of intracellular cyclic AMP levels and the levels of cyclic AMP-independent protein kinases in cytoplasm and nucleus, as a first step in an analysis of the mechanism of Ca^{2+} effects on myogenesis.

MATERIALS AND METHODS

Cell culture. Cultures were prepared from 12-day embryonic chick thigh muscle by mechanical dissociation (4) and grown as described previously (1). Total Ca^{2+} levels in Ca^{2+}-depleted medium (containing dialysed serum and

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embryo extract) were 15-20μM as prepared and 25-30μM at the end of 3 days incubation with cell cultures, as determined on a Unicam SP 90A atomic absorbance spectrophotometer. All cultures were grown for 24h in standard, high Ca^{2+} medium before changing to medium with the appropriate amount of Ca^{2+} added.

Protein kinase assays. Cell pellets were lysed by freezing and thawing in 0.2ml of double glass-distilled water and centrifuged at 2,000g for 10 min. The supernatants were centrifuged again at 10,000g for 10 min. 20μl of supernatant were assayed at 30°C in a final volume of 100μl of assay buffer (60mM sodium phosphate buffer pH 6.4; 0.5mM EGTA; 10mM Magnesium acetate; 20mM NaF; 60mg/ml casein, pretreated according to (5); 1μCi γ-[^{32}P] ATP (16-19 Ci/mmole; Radiochemical Centre, Amersham, Bucks, U.K.); 0.2mM ATP). Reactions were terminated by cooling in iced-water and adding excess 10% trichloroacetic acid with 50mM Na phosphate. Samples were filtered and washed four times with trichloroacetic acid and once with ethanol.

Nuclear protein phosphorylation assays were performed as described previously, with endogenous nuclear protein as substrate (6).

Cyclic AMP assays. Cells were rinsed very rapidly with ice-cold 0.9% NaCl - 1mM EDTA, frozen in situ and harvested in a 4°C cold-room in a small volume of 0.9% NaCl - 1mM EDTA. An aliquot of the resulting cell lysate was taken for protein estimation (7) and the remainder was boiled for 3 min. and centrifuged for 20 min. at 2,000g. Cyclic AMP in the supernatant was determined with an assay kit (Radiochemical Centre) which depends on competition with [^{3}H] cyclic AMP for cyclic AMP-binding protein. Cyclic AMP-phosphodiesterase (Sigma) destroyed over 95% of the cyclic AMP competitor activity in the samples and internal cyclic AMP standards added to samples produced the predicted results in the assay.

RESULTS

Fig. 1A shows that ^{32}P incorporation into casein by cytoplasmic extracts is linear for the first 10 min. and almost linear for a further 20 min. Fig. 1B shows that incorporation is proportional to the amount of cell extract in the assay for both 48h and 96h cultures. This linearity suggests that the incorporation does indeed reflect the amount of protein kinase enzyme in the extract and also that any endogenous cyclic AMP in the extract is not affecting the enzyme activity (saturation with cyclic AMP is most unlikely at the 20-fold dilution used, given that cyclic AMP levels in these cells are not unusually high (8; and later Figs. 4 and 5)).

Fig. 2 shows the dependence of cytoplasmic protein kinase levels on the Ca^{2+} concentration in the cell culture medium. Since we are interested in the possible involvement of cyclic AMP and protein kinases in the muscle-specific protein accumulation which occurs between 24h and 96h of culture, measurements were made during this period at 48h and 72h. There is, at both times, an

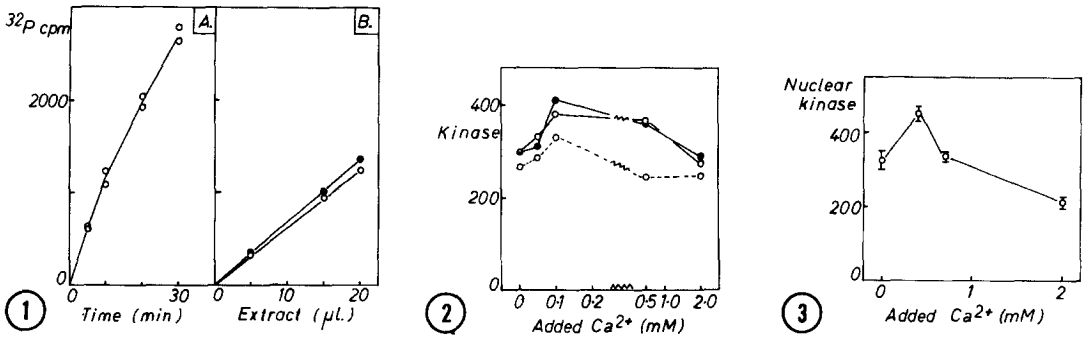


Figure 1A. Time-course of the protein kinase assay. An extract from 96h, 2mM Ca^{2+} cultures was used and zero-time background of 300cpm has been subtracted. Each point is a single determination.

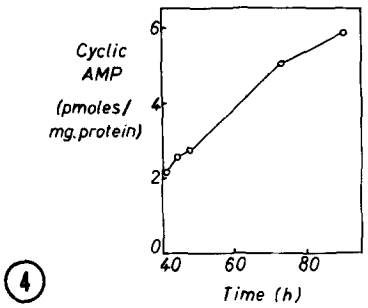
Figure 1B. Effect of dilution of the extract on ^{32}P incorporation. Extracts from 48h (0.1mM Ca^{2+}; open circles) and 96h (0.5mM Ca^{2+}; filled circles) cultures were used in the experiments illustrated. Zero-time backgrounds were subtracted. Each point is the average of triplicate (48h) or duplicate (96h) incubations.

Figure 2. Effect of external Ca^{2+} in the culture medium on the levels of cytoplasmic protein kinase at 48h (o----o) and 96h of culture, and with 10^{-6}M cyclic AMP present (• —— •) or absent (o —— o) in the assay of 96h extracts. Each point is the average of duplicate or triplicate incubations. With 48h extracts, there was also no significant effect of cyclic AMP (not shown). Results are expressed as ^{32}P cpm incorporated per µg of protein in the extract.

Figure 3. Effect of external Ca^{2+} in the culture medium on the levels of protein kinase in nuclei isolated at 72h of culture. Results are given ± S.E.M. of triplicate assays, and expressed as ^{32}P cpm incorporated per µg of nuclear protein.

increased protein kinase level in response to Ca^{2+} up to 100µM and a subsequent gradual decrease up to 2mM Ca^{2+}. There was no significant effect of 10^{-6}M cyclic AMP in the assay of any of the 72h extracts. Over a longer series of experiments, at various culture times and Ca^{2+} concentrations, the average effect of cyclic AMP was a stimulation of 5%, suggesting that, at most, only a minor component of the phosphorylating activity is cyclic AMP-dependent, as described for the nuclear enzyme (9). The protein kinase activity in nuclei isolated from 72h cultures shows a basically similar response (Fig. 3), even though the assays were performed under rather different conditions and are therefore not directly comparable.

Fig. 4 shows the increase in cyclic AMP levels which occurs during differentiation in culture. The pattern and absolute levels are almost



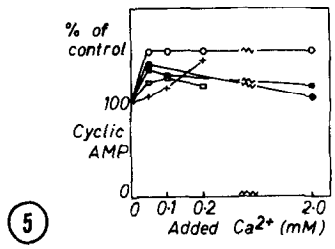


Figure 4. Cyclic AMP levels (p moles / mg of total protein) during myoblast differentiation. Each point is the average of duplicate determinations (range of duplicates $< \pm 5\%$).

Figure 5. Effect of external Ca^{2+} in the culture medium on intracellular cyclic AMP levels. Results are expressed as % of the level in p moles / mg protein in cultures with Ca^{2+}-depleted medium alone (zero added-Ca^{2+}). Each curve represents a different time of harvesting cultures between 48h and 90h (48h: open circles; 55h: crosses; 66h: filled squares; 72h: open squares; 90h: filled circles).

identical to those described by Moriyama and Murayama (10), using quite different assay methods. The absence of the sharp peak of cyclic AMP described elsewhere (11) may simply be due to insufficient time-points during the appropriate culture period. The pattern of response of cyclic AMP levels to external Ca^{2+} (Fig. 5) is somewhat similar to that of protein kinase levels. The effects here are rather variable in magnitude between experiments but a stimulation of cyclic AMP levels by intermediate Ca^{2+} concentrations was always observed.

Comparing these results with effects of external Ca^{2+} on MM-CPK levels (1), it would seem that MM-CKP accumulation is reduced when cyclic AMP and protein kinase are elevated.

DISCUSSION

In an earlier study (1), one hypothetical explanation for Ca^{2+} effects on CPK levels involved Ca^{2+}- and cyclic AMP-dependent protein kinases and required that cyclic AMP be highest at very low Ca^{2+}. The present experiments clearly rule out that precise explanation, though the possibility of cyclic nucleotide involvement in some form has not been eliminated.

Elevated cyclic AMP levels are usually associated with quiescent, as opposed to actively-dividing, cells and, in myoblast cultures, cyclic AMP does indeed increase between 48h and 96h of culture (Fig. 4), when [^{3}H]-thymidine incorporation into DNA and cell division are declining (12). One might expect, therefore, that the rather similar increase in cyclic AMP in response to 0.05-0.2mM extracellular Ca^{2+} (Fig. 5) would bring about a premature decline in [^{3}H]-thymidine incorporation relative to both normal and Ca^{2+}-depleted media. This is not the case, however, since [^{3}H]-thymidine incorporation is actually highest at intermediate Ca^{2+} concentrations (13). In some circumstances, elevated cyclic AMP can be associated with <u>increased</u> cell division, but Berridge (2) suggests that these can be best explained if Ca^{2+} itself is the more direct signal for cell division, cyclic AMP acting by modulating intracellular Ca^{2+}. Even if intracellular Ca^{2+} does mediate external Ca^{2+} effects, the observed changes in cyclic AMP-<u>independent</u> protein kinase levels (Fig.2) would suggest that protein phosphorylation might still play a role.

Correlations between cell division and muscle-specific protein synthesis in myoblast populations have been described, even to the extent that in a given cell they may be mutually exclusive (14). The effects of Ca^{2+} on [^{3}H]-thymidine incorporation (13) might therefore seem sufficient to account for its opposite effects on CPK levels. However, not all muscle-specific proteins show the same pattern of response to external Ca^{2+} as CPK, a notable exception being the cell-surface acetylcholine receptor (15). The data, therefore, do not yet justify the conclusion that Ca^{2+} effects on CPK levels are mediated by cyclic AMP effects on cell division.

The nuclear protein kinase activity may be, in part at least, the same enzyme that phosphorylates casein and both activities are cyclic AMP-independent (16). Although redistribution of proteins between nucleus and cytoplasm may occur during myogenesis (17), it does not appear to be responsible for any of the changes described here, since nuclear and cytoplasmic enzymes show similar changes (Figs. 2 and 3). Leakage of enzyme from the nucleus during preparation

of cytoplasmic extracts has not been ruled out in these experiments, but this would have little effect on the overall conclusions. Contamination of nuclei by cytoplasmic proteins is negligable (17).

The changes in cyclic AMP levels described here (Figs. 4 and 5) are within the concentration range (0.1-1.0μM) likely to affect certain cyclic AMP-dependent protein phosphorylations in intact cells (8). It would seem, therefore, that intermediate external Ca^{2+} concentrations are potentially capable of increasing protein phosphorylation in muscle cells both by increasing cyclic AMP-dependent protein kinase activity through cyclic AMP levels and by increasing the levels of cyclic-AMP independent enzymes. Whether these changes are subsequently responsible for observed changes in muscle-specific protein accumulation in response to Ca^{2+}, however, has not been established.

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