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THE ISOLATION OF NUCLEI FROM *PHYSARUM FLAVICOMUM:* DEMONSTRATION OF A NUCLEAR CYCLIC AMP PHOSPHODIESTERASE

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ABSTRACT

Cyclic AMP phosphodiesterase activity in the nucleus of the myxomycete *Physarum flavicomum* was demonstrated by cytochemical staining utilizing electron microscopy and by enzymatic assays with tritiated cyclic AMP as the substrate. Cytochemical staining showed *Physarum's* plasmodial phosphodiesterase activity to be located in the nucleus, along the plasma membrane, in vesicles, and free in the cytoplasm. Nuclear phosphodiesterase, which may be cell cycle dependent, was primarily located in the nucleolus. Nuclei from three to five day old microplasmodial cultures were isolated by the method of Henney and Yee. Whole cells were collected through centrifugation and washed. Pellets were homogenized in a medium composed of 0.01 M Tris-HC1 (pH 7.2 at 4 °C), 0.25 M sucrose, 0.01% Triton X-100, and 5 mM $CaC1_2$. Nuclei were collected through double filtration and two 1.0 M sucrose density gradient centrifugations. After the nuclei were washed, microscopic examination revealed a purity of over 90%. Radioactive assays of the nuclear preparations demonstrated phosphodiesterase activity consistant with that indicated by cytochemical localization. The specific activity of the nuclear enzyme was 15 nMole of cyclic AMP hydrolyzed /min/mg. of protein.

INTRODUCTION

Adenosine 3′,5′-monophosphate (cAMP) is involved in the modulation of an array of metabolic, growth, and differentiation events (Konijn et al., 1967; Whitfield et al., 1979; Bombik and Burger, 1973; Froehlich and Rachmeler, 1972; Burger et al., 1972). Cyclic AMP functions both as an intracellular and intercellular signal transducer. The level of cAMP in and around cells is regulated by the interplay of the cAMP synthetic enzyme adenylate cyclase and the enzyme responsible for the hydrolysis of cAMP, which is cyclic 3′,5′- nucleotide phosphodiesterase.

Myxomycetes are excellent models for the study of cAMP and its regulatory enzymes. The multistaged life cycle of myxomycetes alternates between diploid and haploid vegetative forms which may reproduce asexually by spore formation or sexually by fusion of haploid cells to form a zygote. The predominate structure in the diploid phase is the plasmodium. The plasmodium is a large single cell that is multinucleated. Each plasmodium contains millions of nuclei and all the nuclei within one plasmodium divide at the same time demonstrating mitotic synchrony. The typical *Physarum* cell cycle takes 10-12 hours, 20 minutes of which are required for mitosis. These events in the plasmodium provide a valuable model for the study of mitotic regulation.

Several molecular forms of intracellular and intercellular phosphodiesterase are found in the various life cycle stages of myxomycetes (Lynch and Farrell, 1984a; 1984b, and 1985). Since phosphodiesterase is the only enzyme known to hydrolyze cAMP, each of these forms may play a role in the function of cAMP in life cycle events. Knowledge of the state specific cellular location of phosphodiesterase will increase our understanding of the role of cAMP in myxomycetes.

This report will describe the electron microscopic localization of phosphodiesterase in the plasmodia of the myxomycete *Physarum flavicomum* and the demonstration of phosphodiesterase activity by the hydrolysis of ^{3}H-cAMP in isolates of purified plasmodial nuclei.

MATERIALS AND METHODS

Microplasmodia of *Physarum flavicomum,* grown in liquid shake cultures (Lynch and Farrell, 1984), were harvested at 5 days by centrifugation at 2500 xg for 10 min. and washed with cold distilled water. Microplasmodia were prepared for electron microscopic localization of phosphodiesterase by the technique of Florendo, et al., 1978. The cells were fixed in 0.2% glutarldehyde buffered with 0.25 M sodium cacodylate at pH 7.4. Following a buffer wash the cells were preincubated for 30 min. at room temperature in a reaction medium containing 80 mM Tris Maleate, 250 mM sucrose, and 3 mM $MgSO_4$, at pH 7.4. This media included 5 mg/ml snake venom from *Crotalus atrox* (as a source of exogenous 5′ nucleotidase) which hydrolyzes all existing 5′ nucleotides that may yield a false reaction product. Free phosphate was removed by washing the cells in reaction medium without snake venom. They were then incubated at 37 °C for 30 min. in reaction media containing 80 mM Tris maleate, 250 mM sucrose, 3 mM cyclic AMP (as an exogenous substrate), 3 mg/ml snake venom, and 2 mM lead nitrate at pH 7.4. This incubation results in the hydrolysis of exogenous cAMP to 5′AMP which is then hydrolyzed to liberate phosphate which in turn reacts with available lead ions to form an electron opaque precipitate at the reaction site. Controls were incubated in reaction medium without 3 mM cyclic AMP as an exogenous substrate. This control demonstrates any non-specific lead-phosphate deposition. After incubation, the cells were rinsed with 0.1 M cacodylate buffer and postfixed with 1% osmic acid for 30 min. The tissues were dehydrated through graded acetone series and embedded in Spurr's epoxy resin (Spurr, 1969). Ultrathin and semithin sections were cut with a Porter-Blum MT2 ultramicrotome, poststained with saturated methanolic uranyl acetate, and examined in the STEM mode on an ISI DS 130 electron microscope.

Microplasmodial nuclei were isolated and purified using the method of Henney and Yee (1979). Cells were homogenized in a media containing 0.01 M Tris-HC1, 0.25 m sucrose, 0.01% Triton X-100 and 5 mM CaC_{12} at pH 7.2 and 4 °C. Nuclei were separated from cellular debris by two successive filtrations through milk filters. Isolated nuclei were disrupted by treatment in a Mickel Disintegrator min at 4 °C. Cyclic AMP phosphodiesterase activity was measured by a well established resin procedure using ^{3}H-cyclic AMP as the substrate (Lynch and Cheung, 1975).

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RESULTS

Cyclic AMP phosphodiesterase activity was ultrastructurally localized in the microplasmodia of *Physarum flavicomum* by the presence of electron opaque lead phosphate precipitate at the enzyme reaction site (Figures 1 and 2). Enzyme activity appeared to be located in four distinct regions; (1) bound to the plasma membrane, (2) free in the cytosol, (3) inside vesicles and (4) in the nucleus. In Figure 1 the enzyme activity is localized in the cytosol and bound to the membrane of cytoplasmic vesicles. No nuclear labeling is seen in these cells. In some cells observed, the label also appears bound to the outside of the plasma membrane. In Figure 2 the enzyme is localized in the cytosol and in the nuclei. The nuclear enzyme appears to be exclusively located in the nucleolus. The staining pattern of the nucleolar enzyme indicates that it exists in both an active and inactive form. Control tissue demonstrated no significant lead deposition.

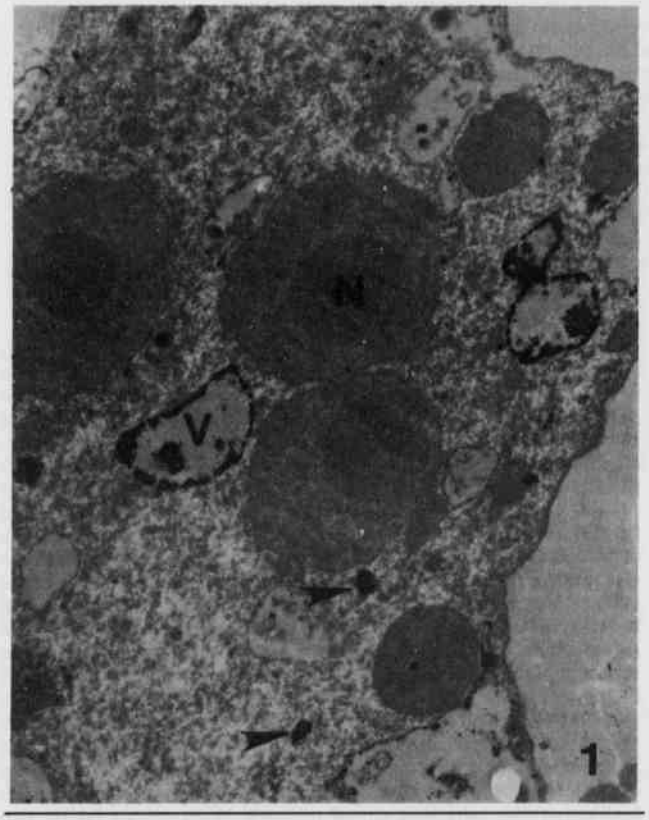

Figure 1. Electron micrograph of cytochemical localization of phosphodiesterase activity in microplasmodium. Dense label is localized in cytoplasmic vesicles (V). Light localization is seen in the cytosol (arrows). The nucleus and nucleolus (N) demonstrate little or no labelling. X 6000

To further demonstrate the presence of a nuclear enzyme, we isolated and purified plasmodial (Figure 3) nuclei and directly assayed these nuclear fractions for phosphodiesterase activity. These nuclei were disrupted with a Mickel Disintegrator and aliquots were assayed for enzyme activity. As seen in Table 1, these isolated nuclear preparations contained enzyme activity of about 15 nMole of cyclic AMP hydrolyzed/min/mg of protein.

DISCUSSION

We previously reported on both intracellular and extracellular

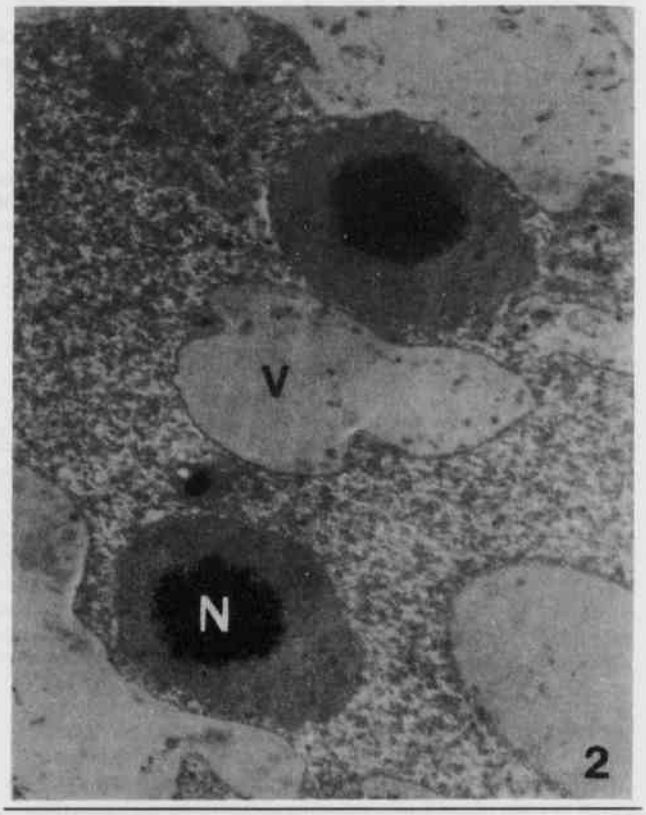

Figure 2. Electron micrograph of cytochemical localization showing intense labelling of phophodiesterase activity in the nucleolus (N). Light labeling occurs in the cytoplasm. No label was noted in cytoplasmic vesicles (V). X 6000

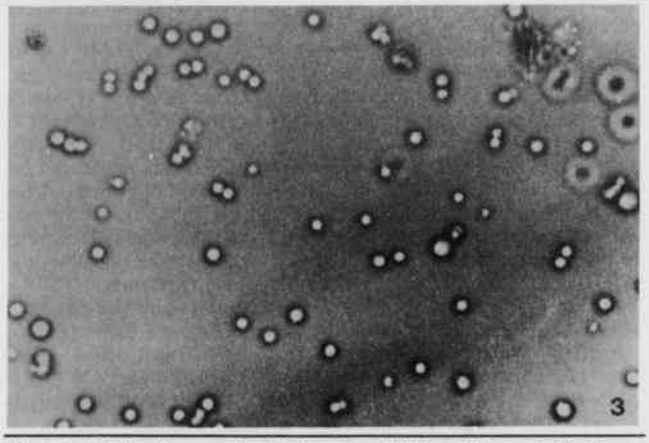

Figure 3. Light micrograph of isolated nuclei diluted fromt he final centrifugation pellet. X 600

phosphodiesterase from the plasmodium of *P. flavicomum*. The intracellular enzyme was most likely a mixture of the various enzymic forms indicated by Figures 1 & 2. Indeed, as indicated by our first publication on this enzyme, we showed that soluble enzyme activity would vary depending on how fast we centrifuged our homogenates (Lynch and Farrell,1984a). The data in this paper suggest that the results may have been due to the heterogenous nature of the enzyme.

The enzyme localized inside vesicles in Figure 1 may indeed be the same extracellular enzyme that we reported on previously. If so, the

Table 1. Cyclic AMP phosphodiesterase activity from isolated, broken nuclei.

Cyclic AMP Phosphodiesterase Activity From Isolated, Broken Nuclei

Sample Size	cpm	nMole/min/mg of protein	
30 µl	4231	13.8	
30 µl	3851	10.4	Average
			15.2
60 µl	6500	17.8	
60 µl	6790	19.0	

enzyme in Figure 1 may represent newly synthesized enzyme that has been packaged into vesicles for subsequent release into the medium.

We have demonstrated the presence of a nuclear enzyme by both cytochemical techniques and also direct enzyme measurements in isolated nuclei. One other report of a nuclear phosphodiesterase in a myxomycete is available for *Physarum polycephalum.* (Kupetz and Jeter, 1985). That data represent only an enzyme assay. Their specific activity ranged from 9 nMole/min/mg protein to 11 nMole/min/mg of protein, which is very similar to that reported in this paper (15 nMole/min/mg of protein).

The pattern of lead deposition for the nuclear enzyme shown here suggests that this enzyme is preferentially activated (or inactivated) at unique time periods in the plasmodium. The variable activity of the nuclear phosphodiesterase and the established role of cyclic AMP in controlling mitosis suggests that cyclic AMP regulated mitosis may occur in these cells. Mitosis is synchronous within each microplasmodium; however, microplasmodia are not in mitotic synchrony with each other. In the same culture flask, each microplasmodium would be in its own stage of the cell cycle. Activation of nucleolar phosphodiesterase may represent a connection of cyclic AMP to specific cell cycle events.

Concrete data for the role of cyclic AMP in the *Physarum* cell cycle is not only sparse but also ambiguous. Some of the first evidence showed great promise (Lovely and Threlfall, 1976, 1978, 1979). Since then, this data has been shown to be equivocal (Garrison and Barnes, 1980; Trakht et al., 1980; Oleinick et al., 1981). The relationship of our study to the above data and the role of a nuclear phosphodiesterased in the cell cycle in *Physarum* is still open for further investigation.

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