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ADENYLATE CYCLASE AND CYCLIC AMP PHOSPHODIESTERASE ACTIVITY DURING THE MITOTIC CYCLE OF *Physarum polycephalum*

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Summary The activity of adenylate cyclase (EC 4.6.1.1.) and cyclic AMP phosphodiesterase (EC 3.1.4.17) were measured in three subcellular fractions of the acellular slime mould *Physarum polycephalum*. Activity of adenylate cyclase was measured using the ATP analogue β-γ imido(8-^{3}H) adenosine 5' triphosphate. Cyclic AMP phosphodiesterase was measured using (8-^{3}H) cyclic AMP as substrate in the presence of endogenous 5'nucleotidase (EC 3.2.2.4.). A particulate fraction showed a peak of adenylate cyclase activity late in G2 whereas other fractions showed no change at any time during the mitotic cycle. No changes in cyclic AMP phosphodiesterase activity were detected at any time in any fraction.

INTRODUCTION During synchronous growth of macroplasmodia of the true slime mould *Physarum polycephalum* we noted a marked but transient peak of cyclic AMP in the last quarter of G2 before mitosis (1). To find out whether this is the result of changes in the activity of adenylate cyclase or of cyclic AMP phosphodiesterase we have now measured the activity of both enzymes. Assay of adenylate cyclase in cell homogenates using ATP as substrate is often complicated by the presence of other enzymes degrading ATP. The activity of these enzymes may be greater than that of the adenylate cyclase. Degradation of ATP may be avoided by using the ATP analogue AMP-PNP (2). Unlabeled (3) and (^{32}P)AMP-PNP (4,5) have been used for this purpose. In our work we introduce the use of (^{3}H)AMP-PNP. To measure cyclic AMP PDE we have used (^{3}H) cyclic AMP as substrate exploiting the presence of an active endogenous 5'nucleotidase in *Physarum* to convert all the 5'AMP to adenosine. In the assay of both enzymes we have used tlc on cellulose to separate substrate from product with monitoring the activity of both by liquid scintillation spectrometry.

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AMP-PNP = β-γ imido adenosine 5'triphosphate, TCA-trichloroacetic acid
cyclic AMP PDE = cyclic AMP phosphodiesterase, IBMX = isobutylmethylxanthine

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MATERIALS AND METHODS (a) Culture of *Physarum* Strain M3C was grown as microplasmodia in a partially defined medium with haemin (6). Macroplasmodia showing mitotic synchrony were set up and cytological examination carried out as described elsewhere (1).

(b) Extraction of enzymes At each sample time one macroplasmodium on filter paper was frozen for 5 min in liquid nitrogen, the filter paper withdrawn and the inoculum discarded. The remaining material was scraped off into four volumes of extraction buffer (0.25M sucrose, 5mM $CaCl_2$, 1mM dithiothreitol, 50mM Tris-HCl pH 7.5) and gently dispersed by 15 strokes in a Potter-Elvejhem homogeniser at 0°C. After centrifugation for 15 min at 10000g at 4°C the supernatant was recentrifuged in a 65 rotor for 2 hr at 100000g at 4°C in a Model L Spinco ultracentrifuge. The pellets from both the low and high speed centrifugation steps were suspended in 2 volumes and 1 volume extraction buffer respectively. Fractions were stored in liquid nitrogen for up to 4 months without loss of activity. For protein analysis 1ml of 10%(w/v) TCA was added to an equal volume of sample and left on ice overnight. The precipitate was collected by centrifugation for 5 min at 2500g and allowed to dissolve in 1ml 0.4M NaOH for 72 hr. Protein was measured by the method of Lowry *et al.*(7) using bovine serum albumin as standard.

(c) Assay of adenylate cyclase To 15µl 0.2mM AMP-PNP (containing approx. 30nCi (^{3}H)AMP-PNP and 15µl assay buffer (5mM $MgCl_2$, 1mM IBMX, 50mM Tris-HCl pH 8.0) was added 15µl homogenate. All assays were run in duplicate. After incubation at 30°C for 10 min the reaction was terminated by addition of 15µl 4N HCl containing, as chromatographic markers, cyclic AMP, 5'AMP and adenosine (approx.5mM). 5µl of the mixture was spotted onto a 20 x 20cm cellulose tlc plate which had been scored into 15mm wide channels. After air drying two more 5µl aliquots were added. Zero time controls had stopping solution added before the enzyme. For the determination of total counts 15µl of the reaction mixture was spotted in the same way onto a 15mm square of a cellulose tlc plate. The plates were developed overnight in a butanol: glacial acetic acid:water mixture (2:1:1, v/v) in pre-equilibriated glass tlc tanks at 4 to 6°C. The plates were air dried and the markers detected under UV (254nm) light. Regions containing cyclic AMP and AMP-PNP were cut out and individually eluted for 20 min with 0.8ml distilled water in plastic scintillation vials. Total count squares were treated in the same way. 8ml scintillant (toluene 1000ml, Triton X-100 500ml,PPO 4.4g, POPOP 0.1g) was added to each vial and the radioactivity measured in a Packard Model C2425 scintillation spectrometer at an efficiency of 30% to better than 5% SD with quenching monitored by use of the external standard channels ratio.

(d) Assay of cyclic AMP PDE To 15µl 0.8mM cyclic AMP (containing approx. 60nCi (8-^{3}H) cyclic AMP and 15µl assay buffer (5mM $MgCl_2$,50mM Tris-HCl pH 7.5) was added 15µl homogenate. After incubation at 30°C for 10 min the reaction was terminated by the addition of 15µl 30%(w/v) TCA containing cyclic AMP, 5'AMP and adenosine (approx 5mM) as chromatographic markers. All assays were run in duplicate. Separation of cyclic AMP, 5'AMP and adenosine by tlc on cellulose, elution and determination of radioactivity were as described in (c) above

(e) Chemicals (8-^{3}H) cyclic AMP (27.5Ci/mmol) and two lots of β-γimido (8-^{3}H) AMP-PNP (20.3 and 15Ci/mmol) were purchased from the Radiochemical Centre, Amersham,Bucks,UK. AMP-PNP and IBMX were bought from the Boehringer Corporation (London) Ltd and the Aldrich Chemical Co respectively. Schleicher and Schuell cellulose (F1440/LS 254) tlc plates were obtained from Andermann & Co Ltd, East Molesey,Surrey.

RESULTS AND DISCUSSION (a) Enzyme assays The Rf values for the substances separated by tlc on cellulose were: AMP-PNP 0.13, cyclic AMP 0.37, 5'AMP 0.26 adenosine 0.64.

TABLE 1 Assay of adenylate cyclase activity in three fractions of a homogenised macroplasmodium of *Physarum polycephalum*.

Fraction	Adenylate cyclase assay			%substrate	Activity*	K_m
	Total cpm	(^{3}H)AMP-PNP cpm	(^{3}H)cAMP cpm	hydrolysed		x 10^{-5}M
10000g pellet	4237	3935	343	8.1	423	16
100000g pellet	4276	4239	30	0.7	90	40
100000g supernatant	4307	4011	306	7.1	229	12

* Activity pmol cyclic AMP/10 min/protein(mg).
Corrected cpm values shown after subtraction of a blank of 63cpm.
For details see Materials and Methods (c).

Under the conditions described in section (c) above the adenylate cyclase reaction was linear for up to 10 min with up to 60µg protein per assay tube for fractions differing in activity by a factor of five. The cyclic AMP produced by the reaction was identified by its Rf value, its quantitative conversion by beef heart phosphodiesterase to 5'AMP and its binding to a specific cyclic AMP binding protein. IBMX was present in the assay mixture to stop degradation of AMP-PNP and cyclic AMP by native phosphodiesterase. At the concentration used IBMX did not interfere with the activity of adenylate cyclase. The mean and standard deviation (SD) of twelve identical assays run in parallel was: total counts 4660 SD 33, AMP-PNP 4382 SD 26, cyclic AMP 284 SD 26.

In the assay of cyclic AMP PDE sufficient 5'nucleotidase was present in all homogenate fractions to convert all 5'AMP to adenosine. This was shown by monitoring the 5'AMP region on all tlc plates; in no sample was any radioactivity present above background.

(b) Some properties of adenylate cyclase More than half the enzyme activity was located in the low-speed pellet (Table 1). K_m values were determined between 30nM and 33µM AMP-PNP for the cyclase in the three fractions of

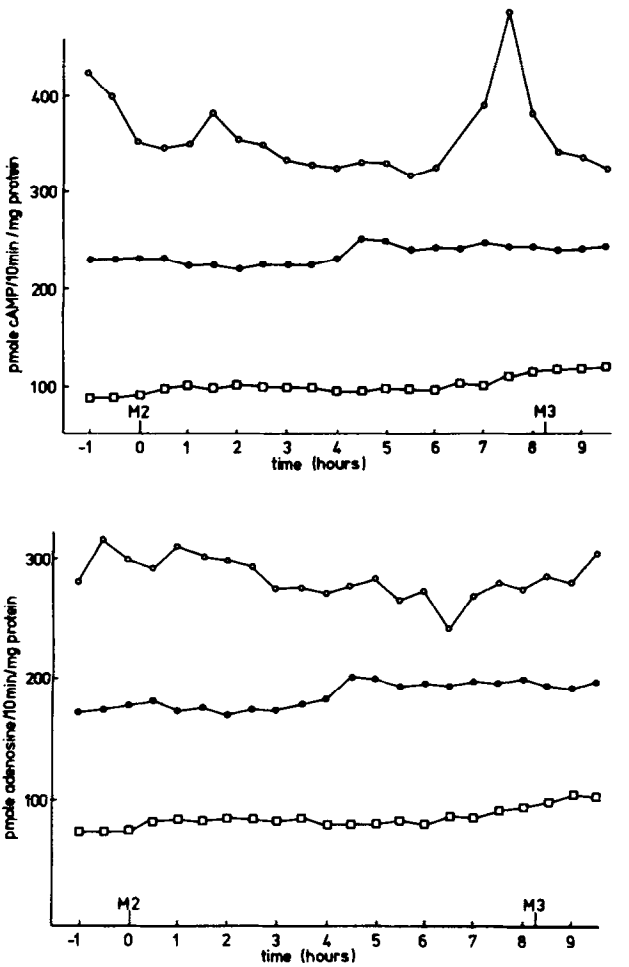

Figure 1 (top) Adenylate cyclase activity in three fractions of a homogenate of a macroplasmodium of *Physarum polycephalum* during the mitotic cycle. M2 second and M3 third metaphase after fusion of microplasmodia. o 10000g pellet, ● 100000g supernatant, ◘ 100000g pellet.

Figure 2 (bottom) Cyclic AMP phosphodiesterase activity in three fractions of homogenised macroplasmodia of *Physarum polycephalum* during the mitotic cycle. Symbols as in Figure 1.

homogenised macroplasmodia are shown in Table 1. For each fraction the optimum pH was 8.0. This is higher than the value of 7.2 used by Atmar *et al.* (8) with ATP as substrate but this may result from the higher pK_a value of the phosphate in AMP-PNP of 7.7 compared with that of 7.1 for ATP (2). There was

no stimulation of activity by 10mM NaF even if the extraction procedure was carried out in the presence of 10mM NaF.

(c) Changes in enzyme activity during the mitotic cycle Figures 1 and 2 show the adenylate cyclase and cyclic AMP PDE activity respectively. During a period a period late in G2 there is a rapid rise and fall in adenylate cyclase activity in the low speed particulate fraction which coincides with a rise and fall in cyclic AMP at this time (1). Other homogenate fractions showed no clear change in enzyme activity. With respect to cyclic AMP PDE we could detect no change in activity during the mitotic cycle thus confirming a previous report (9). In human lymphoid cells a peak of cyclic AMP and adenylate cyclase was seen to coincide in G2, both declining to a low level at mitosis. In the G1 period following mitosis cyclic AMP levels increased (10). In macroplasmodia of *Physarum* mitosis is followed by the S phase during which cyclic AMP remains low. In lymphoid cells cyclic AMP PDE activity is maximal at mitosis when cyclic AMP is low (10); in *Physarum* it would appear that the cyclase enzyme principally regulates the level of cyclic AMP.

The time of peak adenylate cyclase activity in *Physarum* is also coincident with the phosphorylation of F1 histone proteins (11). Whether the increase in enzyme activity at this time results from enzyme synthesis, the unmasking of enzyme precursors, the appearance of a stimulator (or the disappearance of an inhibitor) cannot be decided. Atmar *et al.* (8) have shown a potential role for polyamines in controlling adenylate cyclase activity.

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