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THE ACTIVITY OF GUANYLATE CYCLASE AND CYCLIC GMP PHOSPHODIESTERASE DURING SYNCHRONOUS GROWTH OF THE ACELLULAR SLIME MOULD *Physarum polycephalum*

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Summary Guanylate cyclase (EC 4.6.1.2.) and cyclic GMP phosphodiesterase (EC 3.1.4.-.) activity were measured in three subcellular fractions of *Physarum polycephalum* macroplasmodia isolated at intervals during synchronous growth. In a particulate fraction prepared by high-speed centrifugation guanylate cyclase activity was twice to ten times that of other fractions and highest in mid S and late G2. Two-thirds of the cyclic GMP phosphodiesterase activity was in a soluble fraction but there was no significant change in enzyme activity or distribution during the mitotic cycle.

INTRODUCTION During synchronous growth of the acellular slime mould *Physarum polycephalum* we detected two peaks of cyclic GMP, the larger during S, the other just before mitosis (1). We report here changes in the activity of enzymes responsable for cyclic GMP synthesis and degradation among subcellular fractions of the macroplasmodium isolated at intervals during the mitotic cycle. An account of the relevant enzymes concerned with cyclic AMP metabolism in this organism has been published (2). The formation of cyclic GMP from GTP is catalysed by guanylate cyclase. The possibility that cyclic AMP may be formed from ATP by this enzyme, as has been observed in rat liver (3) has not been explored. GTP is liable to be degraded by phosphatases whose activity may exceed that of the cyclase. To avoid loss of substrate during enzyme assay we have used β-γ imido guanosine triphosphate (GMP-PNP)

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GMP-PNP = β-γ imido guanosine 5' triphosphate.
Cyclic GMP PDE = cyclic GMP phosphodiesterase. TCA = trichloroacetic acid
Cyclic AMP PDE = cyclic AMP phosphodiesterase.
TES = N-tris (hydroxymethyl) methyl-2-aminoethane sulphonic acid.
SQ 20009 = 1-Ethyl-4-(isopropylidenehydrazino)-1H-pyrazolo-(3,4-b)-pyridine-5-carboxylic acid ethyl ester hydrochloride.

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Replacement of oxygen in the β-γ bridge by an -NH group confers resistance to hydrolysis by GTPase at this position without altering its ability to be cleaved by cyclase (4). Using tritiated GMP-PNP separation of substrate from product has been achieved by tlc on cellulose and both estimated by liquid scintillation counting. An inhibitor of cyclic GMP PDE has been included in the assay to restrict degradation of cyclic GMP without changing the activity of guanylate cyclase. In the measurement of cyclic GMP PDE we have allowed the active endogenous 5'nucleotidase (EC 3.2.2.4.) to convert all the 5'GMP to guanosine. Again a radiometric method combined with tlc has been developed.

MATERIALS AND METHODS (a) Culture of *Physarum polycephalum*. Strain M3C was grown as microplasmodia in a partially defined medium with haemin (5). The establishment of macroplasmodia showing mitotic synchrony has been described elsewhere (1).
(b) Extraction of enzymes. Whole plasmodia were frozen in liquid nitrogen, the inoculum discarded and the previously actively growing region placed in four volumes of extraction buffer (0.25M sucrose, 5mM $CaCl_2$, 1mM dithiothreitol, 50mM Tris-HCl pH 7.5) at 0°C. After gentle dispersion in a Potter-Elvejhem homogeniser the suspension was centrifuged 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 pellet from the low speed and high speed centrifugation steps were suspended in two and one volume of extraction buffer respectively and stored in liquid nitrogen for up to 4 months without detectable loss of enzyme activity. For protein analysis 1ml 10%(w/v) TCA was added to an equal volume of sample and left at 4°C overnight The precipitate was collected by centrifugation for 5 min at 2500g and dissolved in 1ml 0.4M NaOH for 72 hr. Protein was measured by the method of Lowry *et al.*(6) using bovine serum albumen as standard.
(c) Assay of guanylate cyclase Duplicate reaction mixtures containing 15μl assay buffer (1mM SQ 20009, 25mM TES-NaOH pH 8.25) and 15μl approx. 6.7mM GMP-PNP (100 to 150 nCi (^{3}H)GMP-PNP) and 15μl homogenate were incubated at 30°C for 10 min and the reaction terminated by the addition of 15μl 1N HCl containing as chromatographic markers approx 5mM GMP-PNP and cyclic GMP. 5μl of the mixture was spotted onto a 20 x 20cm cellulose tlc plate which had been scored into channels 15mm wide. After air drying two more aliquots were added. Zero time controls had 15μl iN HCl added before addition of the homogenate and were then incubated as above. The 'total counts' were determined by spotting 15μl of the reaction mixture in the same way onto a 15mm square of a cellulose tlc plate. Plates were developed in System 1 (2 propanol:dimethylformamide:ethyl methyl ketone:water:ammonia, 20:20:20:39:1 by volume) in pre-equilibrated glass tanks at 5°C. Plates were air dried, examined under UV(254nm) light and regions containing cyclic GMP and GMP-PNP cut out and individually eluted for 20 min with 0.8ml water in plastic scintillation vials. 'Total counts' squares were treated in the same way. 8ml scintillant (Toluene 1000ml, Triton X-100 500ml, POPOP 0.1g, PPO 4.4g) were added to each vial and radioactivity determined in a Packard C2425 scintillation spectrometer at an efficiency of 30% to better than 5% SD with quenching monitored by use of the external standard channels ratio method.

(d) Assay of cyclic GMP PDE To 15μl assay buffer (5mM $MgCl_2$, 50mM Tris-HCl pH 7.5) was added 15μl 6.75 to 8.00mM cyclic GMP containing approx 100 nCi (^{3}H) cyclic GMP and 15μl *Physarum* homogenate. Duplicate assay tubes were incubated at 30°C for 10 min and the reaction stopped by adding 15μl 30%(w/v) TCA containing approx 5mM cyclic GMP, 5'GMP and guanosine as chromatographic markers. Three 5μl aliquots were spotted on cellulose tlc plates as described in (c) and when dry developed in System 2 (95% ethanol:2M ammonia: 2M acetic acid, 100:20:20 by volume). Localization, elution and determination of the radioactivity of separated substances were as described in (c) above.
(e) Chemicals (8-^{3}H) cyclic GMP (19.0 Ci/mmol), (8-^{3}H)GMP-PNP (10.8 Ci/mmol) and cyclic GMP antibody from a kit were bought from the Radiochemical Centre, Amersham,Bucks,UK. Cyclic GMP and GMP-PNP were obtained from the Sigma Chemical Co and Boehringer Corporation London respectively. SQ 20009 was a generous gift of the Squibb Medical Research Institute, New Jersey, USA. Schleicher & Schuell cellulose tlc plates (F1440/LS254) were obtained from Andermann & Co Ltd, East Molesey, Surrey, UK.

RESULTS AND DISCUSSION (a) Enzyme assays Rf values on cellulose with System 1 were GMP-PNP 0.00, cyclic GMP 0.51, 5'GMP 0.00 and guanosine 0.51 and with System 2 were GMP-PNP 0.07, cyclic GMP 0.35, 5'GMP 0.12 and guanosine 0.48. All four substances could be separated by two dimensional chromatography using System 1 in one direction followed by System 2 at right angles.
It was found that when a guanylate cyclase reaction was allowed to proceed for 30 min-three times the usual incubation period-little 5'GMP (0.29%) or guanosine (0.48% of total counts) was detected.

In the guanylate cyclase assay the product of the reaction was identified as cyclic GMP by its Rf value, its ability to bind to a specific antibody and by its susceptibility to purified beef heart cyclic nucleotide phosphodiesterase. SQ 20009 a potent cyclic nucleotide phosphodiesterase inhibitor (7) was selected from seven phosphodiesterase inhibitors tested because at the concentration used inhibition of cyclic GMP PDE was 82% with no measurable effect on guanylate cyclase. Over 98% of the counts applies were eluted from the cellulose while cross contamination was less than 0.017%. The pH of 8.25 was found to the optimum value for all fractions. Zero time values of about 50cpm were subtracted from the values obtained with active enzyme. The reaction was found to be linear for up to 20 min and with up to 45μg protein per assay.
Under the conditions described for the cyclic GMP PDE assay the reaction was linear for up to 15 min with less than 45μg protein per tube.

(b) Some properties of the enzymes Using the 100000g pellet as the source of guanylate cyclase with an activity of 163pmole cyclic GMP/10 min/mg protein the effect of Ca^{2+}, Mg^{2+} and Mn^{2+} was investigated over the range 0.01 to 100mM. Calcium and magnesium had little effect showing a range in activity of 76%(1mM) to 110%(0.01mM) for the former and 86%(0.01mM) to 114% (0.1mM) for the latter. Enzyme activity was progressively reduced by increasing concentrations of manganese being only 36% for 100mM Mn^{2+}. In many mammalian tissues guanylate cyclase exists in two forms, particulate and soluble, with different responses to Ca^{2+} and Mg^{2+} (8-10). The possibility of more than one enzyme existing in *Physarum* has not been examined.

No cyclic GMP PDE could be detected in the growth medium whereas cyclic AMP PDE was found both in the growth medium and in the plasmodium (2,11).

(c) Enzyme fluctuation during the mitotic cycle The results of enzyme assays on the three fractions prepared from homogenised plasmodia harvested at intervals during the mitotic cycle are shown in Fig 1 and 2. Guanylate cyclase activity (Fig 1) was greatest in the 100000g pellet and showed maximum activity during S and late G2 when it was more than twice that in the early part of G2 and at least eight times that at mitosis. These peaks in cyclase activity coincide with those in cyclic GMP during the mitotic cycle (1). Guanylate cyclase activity in the other two fractions was about equal and showed no changes comparable in magnitude with that in the 100000g pellet. These results suggest that in this organism guanylate cyclase is principally associated with particulate, possibly membrane, material as in sea urchin sperm (12).

Fig 2 shows that more than half the total cyclic GMP PDE activity was located in the high speed supernatant but neither this fraction nor the others showed any marked change during the mitotic cycle and thus behave in this respect like the cyclic AMP PDE (2). In *Tetrahymena pyriformis* a ten fold change in cyclic GMP PDE was found during one cell cycle (13).

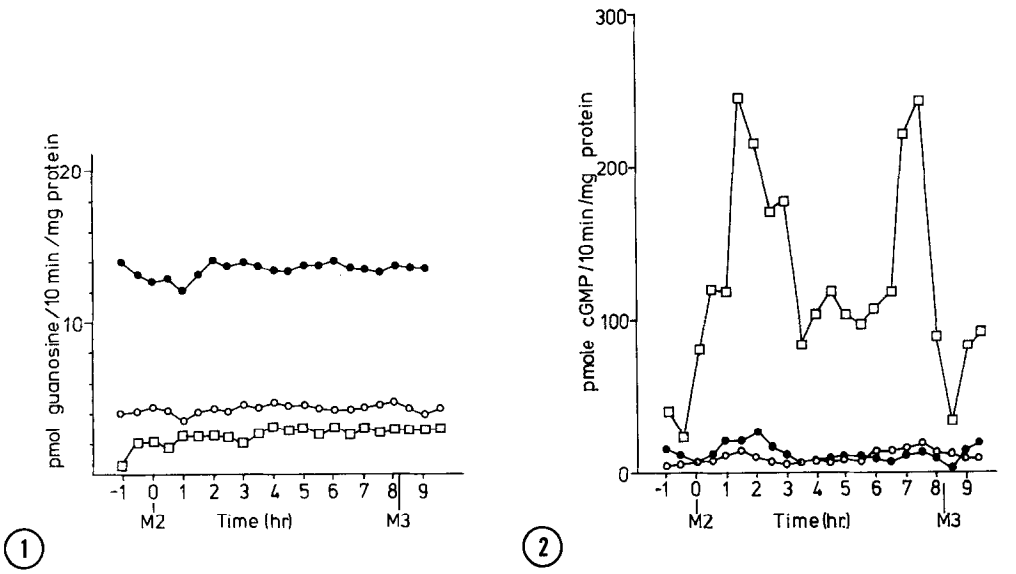

Fig 1 Guanylate cyclase activity in fractions of an homogenate of *Physarum* macroplasmodium during the mitotic cycle. M2 second and M3 third synchronous mitosis respectively after fusion of microplasmodia (See Methods). 10000g pellet —o— 100000g pellet —□— 100000g supernatant. —●—

Fig 2 Cyclic GMP phosphodiesterase activity in fractions of an homogenate of *Physarum* macroplasmodium during the mitotic cycle. See legend to Fig 1 for details.

It seems likely that in *Physarum* the level of cyclic GMP is principally regulated by synthesis.

Both cyclic AMP and cyclic GMP have been implicated in the control of cell proliferation (14). In *Physarum* a striking correlation has been noted between the level of cyclic GMP and the conversion to the A form of ornithine decarboxylase (EC 4.1.1.17) during synchronous growth (15). It is not known whether this enzyme is a phosphoprotein and although cyclic GMP dependent protein kinases have been described (16-18) their presence in *Physarum* has not been reported.

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