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Formation of Cyclic Adenosine-3′,5′-monophosphate (Cyclic AMP) by a Crude Membraneous Fraction of Ehrlich Mouse Ascites Tumour Cells

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By using ion exchange chromatography it has been possible to demonstrate that a membrane preparation of Ehrlich mouse ascites tumour cells can form cyclic AMP. Amounts as small as 2 – 5 picomol of ^{14}C-labelled adenosine-3′,5′-monophosphate could be determined.

It has recently been shown that different types of tumour cells are capable of forming extracellular adenosine triphosphate (ATP) when incubated in a medium containing all the necessary substrates and cofactors of the first energy yielding step of glycolysis.[1,2] The metabolic significance of the ATP forming process at the surface of the cellular membrane is not clear at present. Some of the terminal phosphoryl groups of the extracellular ATP can be transferred into phosphorylseryl and phosphorylthreonyl residues of membraneous proteins.[3,4]

Another possible function of ATP at the membrane could be as a substrate for adenyl cyclase for the formation of cyclic AMP which is perhaps one of the most important regulating factors in the control of a number of cellular processes.[5,6] Adenyl cyclase seems to be particulate and behaves like a lipoprotein.[7] It occurs in most of the highly differentiated animal cells so far studied, with the exception of non-nucleated erythrocytes.[6] In contrast the presence of the enzyme in dedifferentiated human cells has been very little studied. For that reason it was of interest to find out if the enzyme was present in a membrane preparation from Ehrlich ascites tumour cells. Such cells have a high ATP-forming capacity located at the cell surface.[1,2] The level of cyclic AMP depends on the relative activity of at least two enzymes. The first is adenyl cyclase which catalyzes the conversion of ATP into cyclic AMP and pyrophosphate. The second enzyme is a phosphodiesterase which catalyzes the hydrolysis of cyclic AMP into adenosine-5′-monophosphate (AMP).[8,9] Both enzymes are magnesium ion dependent.[7–10]

Measurement of the formation of cyclic AMP by tumour cells required a sensitive and specific assay for this nucleotide. Since the discovery of cyclic AMP several methods for assaying the substance have been reported.[7,11–16] These assays have varying degrees of sensitivity and specificity. None of these was suitable for our purpose. A time-saving, sensitive and specific chemical assay for ^{14}C-labelled cyclic AMP was therefore developed.

MATERIAL AND METHODS

Preparation of membrane material. A hyperdiploid strain of Ehrlich ascites tumour cells (stem ELD, grown in mice), was immediately cooled to 4°C after removal from the peritoneal cavity. All preparations were performed at 4°C when not otherwise stated.

The fresh tumour cells were washed three times with 10 vol. of a medium containing sodium chloride (13×10^{-2} M), potassium chloride (25×10^{-3} M) and finally, to stabilize the cell suspension Dextran (1.5 %, w/v, Dextran T 40, $M_w = 41\,800$; $M_n = 25\,700$; $[\eta] = 0.210$; AB Pharmacia, Uppsala, Sweden).[19] After each washing the cell suspension was centrifuged at 550 *g* for 10 min. After each centrifugation the supernatant was discarded and the packed cells were again suspended in 10 vol. of the same medium. Finally the supernatant was discarded and the cells were suspended in 5 vol. of 0.25 M sucrose.

Such cell suspensions were immediately homogenized in the following way. A Potter-Elvehjem tube was used and the cells were homogenized with a Teflon pestle gently rotating in the tube for 1.5 min. The suspension was centrifuged at 2000 *g* for 20 min and the supernatant was decanted and discarded. It has been found that most of the adenyl cyclase activity of disintegrated cell preparations was recovered in particulate matter which sedimented with relatively low gravitational forces. This fraction is reported to contain nuclei and cell membranes.[7]

The precipitate was suspended in 2 vol. of 2×10^{-2} M glycylglycine buffer, pH 7.8, in 10^{-3} M $MgSO_4$ and homogenized for 20 sec. The suspension was again diluted with 2 vol. of the buffer solution, homogenized for 10 sec and centrifuged at 2000 *g* for 20 min. The supernatant was decanted and discarded. The second precipitate was suspended in 4 volumes of the glycylglycine buffer, homogenized for 5 sec and centrifuged as before. The supernatant was discarded and the precipitate was suspended in a volume of the glycylglycine buffer equal to the precipitate volume. This suspension was lyophilized and the material was stored at −16°C.

Incubation procedure. Before the incubation procedure, partly described by other authors,[7,17] weighed amounts of the lyophilized material were suspended in 0.4 ml of 2×10^{-2} M glycylglycine buffer, pH 7.8, in 10^{-3} M $MgSO_4$ and 1.25×10^{-3} M glutathione (reduced form). The suspension of the material was made in the incubation tubes which were placed in an ice bath until used. Additions were made with the tubes standing in an ice bath in order to avoid reactions other than those during the incubation.

To the material suspension was added 0.4 ml of 0.06 M Tris HCl buffer, pH 7.8, containing [8-^{14}C]adenosine-5′-triphosphate, ammonium salt (6.25×10^{-5} M, specific activity 40 mCi/mmol; Radiochemical Centre, Amersham), caffeine (2×10^{-2} M), $MgSO_4$ (10^{-2} M), KCl (7.5×10^{-2} M), phosphoenolpyruvate (5.8×10^{-3} M), pyruvate kinase (ATP:pyruvate phosphotransferase, E. C. 2.7.1.40; Boehringer & Soehne, Mannheim, West Germany) (20 μg) and NaF (2×10^{-2} M). The total incubation volume was 0.8 ml.

It could be expected that the membraneous preparation contained ATPase activity.[7] Therefore phosphoenolpyruvate, pyruvate kinase and KCl were added in order to regenerate ATP from the adenosine diphosphate (ADP) produced by the enzymatic hydrolysis of ATP, thus keeping the ATP concentration constant throughout the experiment.

Fluoride has been shown to stimulate the adenyl cyclase activity in disintegrated cell preparations from a variety of sources and caffeine was used as an inhibitor of the phosphodiesterase reaction.[7] Since caffeine was much more readily soluble in the incubation medium than theophylline this substance was used in the experiments.

Incubations were carried out at 30°C for varying times in a metabolic shaker. Some tubes with material suspensions were inactivated by placing the tubes in boiling water for 3 min before addition of the Tris HCl buffer with labelled ATP. These tubes were used as blanks.

The incubations were terminated by immediately cooling the tubes in an ice bath and 300 μg of unlabelled cyclic AMP together with 300 μg of AMP were added to each tube. The tubes were heated in a boiling-water bath for 3 min and centrifuged at 550 *g* for 10 min.

Assay of ^{14}C-labelled cyclic AMP. The supernatant obtained was separated by ion exchange chromatography. 0.5 ml of the supernatant was transferred to a Dowex I-X2 (200 – 400 mesh) formate column (0.7 × 10 cm). Elution was performed in two steps. The column was first eluted with 45 ml of water and then with 0.125 M formic acid. Fractions of 2.8 ml volume were collected at 10 min intervals and the optical density was read at 260 nm. Caffeine, adenine, and adenosine were all found to be quantitatively eluted with water. AMP and cyclic AMP were eluted with 0.125 M HCOOH and the separation was satisfactory as shown by Fig. 1. ADP and ATP were eluted with 4 M HCOOH + 0.2 M ammonium formate and 4 M HCOOH + 0.4 M ammonium formate, respectively.

The radioactivity of the chromatographic fractions was measured by adding a 1 ml aliquot to a glass vial with 5 ml dioxane containing 5 mg 2,5-diphenyloxazole and 100 mg naphthalene/ml and counting the mixture in a Beckman LS-250 Liquid Scintillation System for 10 min.

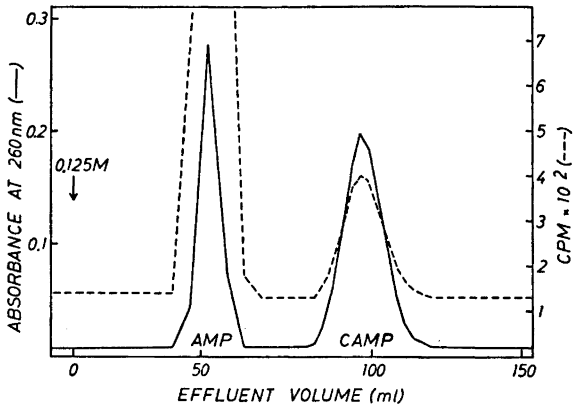

Fig. 1. Separation of AMP and cyclic AMP on a Dowex I-X2 (200 – 400 mesh) formate column (0.7 × 10 cm). The column was eluted first with water and then with 0.125 M formic acid. ———, Absorbance at 260 nm; - - -, ^{14}C-activity (cpm).

Recovery of the assay. In order to estimate the recovery of the analytical procedure separate incubations were performed. Incubations were made as described above, except for the use of unlabelled ATP instead of ^{14}C-labelled. Known amounts of tritium labelled cyclic AMP (^{3}H-3′,5′-AMP, 35 500 cpm; Radiochemical Centre, Amersham), were applied to each incubation tube after the incubations were terminated by boiling. After centrifugation and ion exchange chromatography as described above the eluted activity was measured in the liquid scintillation counter. The recovery ranged from 81 to 89 % with a mean of 84 % (5 analyses). The ^{14}C-cyclic AMP counts from the incubation with ^{14}C-labelled ATP were all corrected to 100 % by use of the recovery data obtained from incubations with ^{3}H-labelled cyclic AMP.

Identification of cyclic AMP. ^{14}C-labelled cyclic AMP was identified by descending paper chromatography on a 60 × 30 cm piece of Whatman No. 1 filter paper at 20°C for 27 h, using a solvent of absolute ethanol/0.1 M boric acid (3.5/1) adjusted to pH 4.0 with glacial acetic acid. The paper was not cut into channels but was used as a whole sheet. This chromatography was a modification of a method previously described by Streeto and Reddy.[17] In the present system cyclic AMP was separated from the following substances tested: ATP, ADP, AMP, adenosine, adenine, and caffeine (Fig. 2).

After drying the paper the nucleotide spots were recognized with UV light. Radioautograms were taken by placing the dried chromatograms in direct contact with the film surface of Kodak Kodirex X-Ray film in darkness for 3 weeks. A well defined spot was observed on the autoradiogram. This shadow coincided with the UV-absorbing spot of cyclic AMP on the paper chromatogram (Fig. 2).

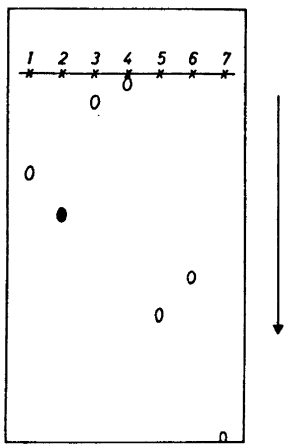

Fig. 2. Descending paper chromatography with absolute ethanol/0.1 M boric acid (3.5/1), pH 4.0 at 20°C for 27 h on Whatman No. 1 filter paper. The black spot indicates the shadow of ^{14}C-labelled cyclic AMP on the autoradiogram. Numbers of reference substances: 1 = AMP, 2 = cyclic AMP, 3 = ADP, 4 = ATP, 5 = adenine, 6 = adenosine, 7 = caffeine.

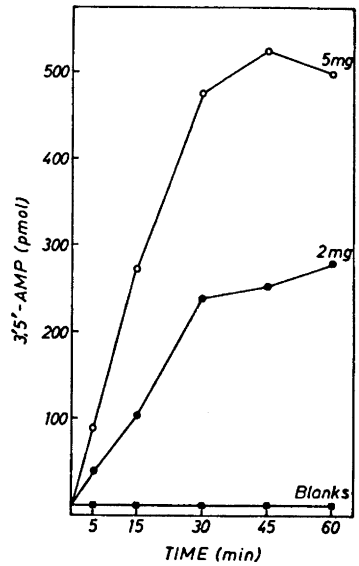

Fig. 3. Formation of cyclic AMP by two different amounts of membraneous material from Ehrlich mouse ascites tumour cells incubated for different times at 30°C. Further details are given in the text.

Sensitivity of the assay. This method with the use of ^{14}C-labelled ATP as a precursor of cyclic AMP is highly sensitive which is shown by the fact that picomol amounts of cyclic AMP could be assayed. The method could easily be made more sensitive by use of ^{14}C-ATP with still higher specific activity.

RESULTS

The results of the incubations indicate a significant adenyl cyclase activity of crude membraneous fractions of Ehrlich ascites tumour cells (Fig. 3). As far as is known there is no report dealing with adenyl cyclase activity of such dedifferentiated human cells. The rate of formation of cyclic AMP is almost linear with time up to 30 min for both 2 mg and 5 mg of enzyme material and also proportional to the amounts of material. The heat inactivated material (blanks) shows no detectable activity.

After incubation for more than 30 min with both amounts of material the curves show a distinct decrease. These declinations in activity seem not only to depend on the fact that the substrate concentrations have reached critical low levels. The activities decrease after the same incubation time for both amounts of material despite more ATP being consumed by 5 mg of active material. The explanation of these concomitantly decreasing activities is uncertain.

DISCUSSION

A proposed model for the adenyl cyclase complex by Robison *et al.*[6] consists of two types of subunits localized in the cell membrane. A regulatory subunit (receptor) faces the extracellular fluid and a catalytic subunit has its active center directed toward the interior of the cell. In this case the substrate for adenyl cyclase should be ATP of intracellular origin. However, ATP formation at the surface of the cell membrane has recently been reported.[1] Thus, it might be necessary to reckon that both the intracellular ATP as well as ATP synthesized in reactions at or in the cell membrane may all provide the adenyl cyclase with the immediate substrate.

Ehrlich ascites tumour cells are highly dedifferentiated and do not necessarily function as target cells for any hormone. Since ascites tumour cells are able to form cyclic AMP it is reasonable to suggest another primary function of this compound in the membrane. Recently, Rasmussen and Tenenhouse [20] claimed that Ca^{2+} and ATP play key roles in maintaining membrane structure, and that the conversion of the membrane from one state to the other is thought to involve a transition from a Ca^{2+}-associated (ATP-Ca^{2+} complex) to a Ca^{2+}-dissociated state. They also concluded that the conversion of ATP to cyclic AMP within the membrane converts the adenine nucleotide from a strong chelator of Ca^{2+} to an extremely weak one, thereby leading either to the release of Ca^{2+} or to the possible complexing of Ca^{2+} by other groups, *e.g.* phospholipids, in the membrane. This would explain, according to the authors, why adenyl cyclase is of necessity a membrane-bound enzyme. The implication is that a small pool of membrane-ATP is the immediate substrate for adenyl cyclase. Friedmann and Park [21] demonstrated that cyclic AMP caused an immediate efflux of Ca^{2+} from the perfused liver of normal and adrenalectomized rats and that this effect preceded an efflux of K^+. Enhancement of phosphoryl transfer by cyclic AMP in the presence of a membraneous fraction from canine kidney was observed by Cunningham.[22] These results indicate a role of cyclic AMP also as a regulator of more primary cell functions not necessarily mediated by hormones.

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