A Simple Assay for Adenylate Cyclase in Intact Cells by Affinity Elution Chromatography

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A method using the principle of affinity elution chromatography is described for the assay of adenylate cyclase in intact human platelets. By incubating platelet-rich plasma in the presence of radioactively labelled adenine, the ATP pool of the cells was prelabelled. Formation of labelled cyclic AMP from ATP was determined by extracting the platelets with HClO_4 . After removal of the latter as KClO_4 , the extract containing cyclic AMP and other adenine nucleotides was adsorbed in a *N,N*-diethyl-*N*-2-hydroxypropylamino (QAE)-cellulose column. The column was washed, and subsequently cyclic AMP was specifically eluted with a cyclic AMP-dependent protein kinase and the radioactivity of the eluate was determined.

Adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] by catalysing the production of cyclic AMP from ATP plays a major role in the mediation of the effects of hormones and membrane-active agents in different cell systems (Rall & Sutherland, 1962; Sutherland *et al.*, 1965; Butcher, 1966). Although this enzyme can be assayed in disrupted cells, it is frequently desirable to determine adenylate cyclase activity in intact cells. In the frequently used method of Kuo & DeRenzo (1969), the metabolic ATP pool is labelled by preincubating the cells in the presence of radioactive adenine and the radioactive cyclic AMP formed is fractionated and assayed by the method of Krishna *et al.* (1968). In these procedures univalent cyclic AMP is separated from bi- and ter-valent adenine nucleotides by sequential chromatography on Dowex (H^+ form) resin and $\text{ZnSO}_4/\text{Ba}(\text{OH})_2$ precipitation. The reliability of the method depends on the assumption that cyclic AMP is the only univalent adenine nucleotide formed from ATP. However, in intact cells, labelled ATP undergoes extensive metabolic changes during the period of incubation and the formation of cyclic AMP may require validation by some other procedure, such as chromatography (Kuo & DeRenzo, 1969) or protein kinase binding (Sinha *et al.*, 1977). Using cyclic AMP-dependent protein kinase, we have developed a simple affinity elution assay to follow the conversion by adenylate cyclase of ATP into cyclic AMP in intact cells and applied it to human blood platelets.

Methods and Materials

Principle

The cellular ATP pool is prelabelled by incubating the cells in the presence of $[^3\text{H}]$ adenine and the

formation of radioactive cyclic AMP from the labelled ATP is determined in HClO_4 extracts of the cells. The radioactive cyclic AMP and other adenine derivatives are adsorbed on a strong anion-exchange cellulose column. After washing the column, the cyclic nucleotide is specifically eluted by cyclic AMP-dependent protein kinase and the radioactivity of the eluate is determined.

Preparation of platelet-rich plasma from human blood

All blood donors had fasted for 12 h and had abstained from medication for at least 2 weeks before blood donation. Venous blood was collected through silicone-treated needles into plastic syringes and anticoagulated by mixing 9 vol. of blood with 1 vol. of 3.8% trisodium citrate (final concentration 0.013 M). Platelet-rich plasma was obtained by centrifugation of the samples for 10 min at 170g. The supernatant platelet-rich plasma (3×10^8 – 4×10^8 cells/ml) was collected. Platelet counts were performed in a Coulter counter (model Zb).

Preparation of platelets labelled with $[^3\text{H}]$ adenine

$[2\text{-}^3\text{H}]$ Adenine ($1 \mu\text{Ci}$; 15 Ci/mmol) was added to each 1 ml of the suspension platelet-rich plasma and unless otherwise stated was incubated at 37°C for 2 h with occasional mixing by inversion.

Extraction of nucleotides from the prelabelled platelets

For this, 4 vol. of prelabelled platelet-rich plasma was mixed with 1 vol. of 25% (w/v) HClO_4 (final concn. 5%), vigorously shaken for 5 min, centrifuged at 1530g for 5 min at 23°C and the supernatant was collected. The HClO_4 extract was then neutralized

with 1.0M-KOH with phenolphthalein as an indicator. After cooling to 0°C for 30min, the precipitated KClO_4 was separated by centrifugation at 991g at 0°C and the supernatant was collected for the determination of radioactive cyclic AMP.

Measurement of the formation of labelled cyclic AMP

To 1 ml of the neutralized HClO_4 extract of the platelet-rich plasma, 1 ml of 20mM-sodium acetate/acetic acid buffer, pH 4.0, was added and applied to a column (packed vol. 1 ml) of QAE-cellulose (*NN*-diethyl-*N*-2-hydroxypropylaminocellulose), previously equilibrated with 10mM of the same buffer. The QAE-cellulose column was prepared by using a 1% (w/v) suspension of QAE-cellulose in a short Pasteur pipette plugged with a small piece of glass wool. After the sample was completely adsorbed into the column, it was washed with 5 ml of 10mM-sodium acetate buffer, followed by 0.4 ml of the same buffer containing 20 μg of protein kinase and 28 μg of protein kinase inhibitor and the eluates were discarded. After incubation at 23°C for 3 h, the column was eluted with 1.5 ml of the latter buffer directly into scintillation vials containing 15 ml of scintillation solvent (ACS; Amersham/Searle, Arlington Heights, IL, U.S.A.). The radioactivity was determined in a Packard Tri-Carb liquid-scintillation counter with 45% efficiency.

Cyclic AMP phosphodiesterase treatment of the HClO_4 extract

The neutralized HClO_4 extract (1 ml) adjusted to contain 5mM-imidazole/HCl buffer, pH 7.5, and 2.0mM- MgSO_4 was incubated with 0.04 unit ($\mu\text{mol}/\text{min}$) of phosphodiesterase (0.2 mg of protein) at 30°C for 10 min. The reaction was terminated by the addition of HClO_4 , and after neutralization of the acid with KOH, the phosphodiesterase-treated extract was adsorbed on a QAE-cellulose column and subsequently eluted with protein kinase as described above.

Chromatography

To identify the labelled compound eluted with protein kinase the nucleotides were extracted with HClO_4 as indicated above and were subjected to two-dimensional t.l.c., in the system of Chivot *et al.* (1974) with water as the first solvent and propan-2-ol/satd. $(\text{NH}_4)_2\text{SO}_4/\text{water}$ (2 : 79 : 19, by vol.), pH 4.7, as the second solvent.

Materials

Cyclic AMP-dependent protein kinase (EC 2.7.1.37), cyclic AMP phosphodiesterase (EC

3.1.4.17) and protein kinase inhibitor were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [$2\text{-}^3\text{H}$]Adenine (15 Ci/mmol), [$2\text{-}^3\text{H}$]adenosine (15 Ci/mmol), [$2,8\text{-}^3\text{H}$]ATP (23 Ci/mmol), [$\text{U-}^{14}\text{C}$]ADP (400 mCi/mmol), [$2\text{-}^3\text{H}$]AMP (20 Ci/mmol), cyclic [$8\text{-}^3\text{H}$]AMP (27.5 Ci/mmol) and cyclic [$8\text{-}^3\text{H}$]GMP (30 Ci/mmol) were the products of Amersham/Searle. Prostaglandin E_1 was kindly provided by Dr. J. Pike (Upjohn Co., Kalamazoo, MI, U.S.A.). QAE-cellulose (Cellex QAE) used in these studies was purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.). This ion exchanger is a strongly basic *NN*-diethyl-*N*-2-hydroxypropylamino derivative of cellulose with an exchange capacity of 0.55 mequiv./g.

Results

Elution of cyclic AMP and other adenine compounds by protein kinase

Elution of radioactive cyclic AMP by protein kinase from the QAE-cellulose column was directly proportional to the concentration of the nucleotide (Fig. 1). In different experiments, 2 μCi of ATP (2 μmol), ADP (2 μmol), AMP (2 μmol), cyclic GMP (2 nmol), adenine (0.4 mmol) or adenosine (0.4 mmol) was each substituted for the radioactive cyclic nucleotide. Approx 0.22% of the total radioactivity was eluted with protein kinase with ATP, and less than 0.1% with the ADP, AMP and cyclic GMP. Higher elution of radioactivity with ATP was due to the presence of cyclic [^3H]AMP as contaminant in the [^3H]ATP used. The identification of cyclic AMP was performed by treating it with phosphodiesterase (Sinha & Colman, 1977a). Both adenine and adenosine were not adsorbed to the QAE-cellulose column. However, the radioactive contaminants present in these commercial radiolabelled preparations were adsorbed and eluted by protein kinase and gave rise to approx. 0.01% of the total radioactivity.

Kinetics of the formation of basal cyclic [^3H]AMP pool in platelets

Formation of radiolabelled cyclic AMP could be detected as early as 15 min, and was linear with time for the first 60 min and reached a maximum at about 120 min of incubation at 37°C. The maximum value once attained remained steady at least for 3 h (Fig. 2). Parallel experiments were performed by treating the extracted radioactive materials from the platelets with cyclic AMP phosphodiesterase. No radioactivity was eluted when the phosphodiesterase-treated HClO_4 extracts were adsorbed on the QAE-cellulose columns and subsequently eluted with protein kinase (Fig. 2). Two-dimensional t.l.c. of the

Fig. 1. Elution of cyclic [³H]AMP by protein kinase. Various concentrations of radioactive cyclic AMP were adsorbed on a 1ml QAE-cellulose column. Elution with protein kinase was done as described in the Methods and Materials section. The results represent the mean $\pm$ 2 S.E.M. of three separate experiments each run in triplicate.

Fig. 2. Kinetics of formation of cyclic AMP in human platelets.

Platelet-rich plasma was incubated for various periods at 37°C with [³H]adenine. At the indicated time platelets nucleotides were extracted with HClO₄. After the removal of the acid, the extract was absorbed on a QAE-cellulose column. Radioactive material was eluted by protein kinase with (—) or without (---) phosphodiesterase treatment. The data shown are mean $\pm$ 2 S.E.M. of four different experiments each run in triplicate.

material eluted by protein kinase confirmed the material to be cyclic AMP (results not shown).

Stimulation of formation of radiolabelled cyclic AMP by prostaglandin E₁

Since prostaglandin E₁ is a potent stimulator of adenylate cyclase (Wolfe & Shulman, 1969), its effects were tested on formation of cyclic AMP in intact platelets by this method. As little as 25 nm-prostaglandin E₁ stimulated the formation of cyclic AMP 2-fold (Table 1). Almost 5-fold stimulation of the formation of cyclic AMP was noted in the presence of a higher concentration of prostaglandin E₁ (100 nm). Addition of theophylline (5 mM), an inhibitor of phosphodiesterase, to the medium potentiated the effect of both concentrations of prostaglandin E₁ on the conversion of radioactive ATP into cyclic AMP.

Discussion

Protein kinase binding has been extensively used for the assay of cyclic AMP in biological materials (Walton & Garren, 1970; Gilman, 1970; Brown *et al.*, 1971; Sinha & Colman, 1977b). However, in these previously described procedures, various nucleotides,

Table 1. Stimulation of cyclic AMP formation by prostaglandin E₁ and theophylline

Platelet-rich plasma (1.0ml) was prelabelled with [³H]adenine for 120 min at 37°C. After prelabelling, the platelet-rich plasma was further incubated for 2 min in the presence or absence of 5 mM-theophylline. After the second incubation, prostaglandin E₁ was added to the incubation mixture as shown. The platelets were incubated for 30 s in the presence of the hormone and then extracted with HClO₄. The HClO₄ extract was neutralized with KOH and after cooling the precipitated KClO₄ was removed by centrifugation. To 1 ml of the neutralized HClO₄ extract 1 ml of 20 mM-sodium acetate/acetic acid buffer was added and passed through 1.0 ml of QAE-cellulose equilibrated with 10 mM-sodium acetate/acetic acid buffer, pH 4.0. The column was then washed and the elution was performed with 0.4 ml of the same buffer containing 20 μ g of protein kinase and 28 μ g of protein kinase inhibitor. After incubation at 23°C for 3 h, the column was eluted with 1.5 ml of the above buffer and the radioactivity was determined as described in the Methods and Materials section. The data represent means $\pm$ 2 S.E.M. for three experiments performed in triplicate.

Addition	Radioactivity of the eluted materials (c.p.m.)	
	No theophylline	5 mM-Theophylline
Prostaglandin E ₁ (nm)		
0	406 $\pm$ 46	562 $\pm$ 31
25	1021 $\pm$ 78	1375 $\pm$ 70
100	1906 $\pm$ 46	2656 $\pm$ 48

including ATP, compete with cyclic AMP for protein kinase binding and thus these methods cannot be used for the assay for the conversion of labelled ATP into cyclic AMP. Since only a small fraction of the total ATP is converted into cyclic AMP, presence of excess radioactive ATP in the protein kinase binding of labelled cyclic AMP would severely interfere with the assay. In the affinity elution procedure, ATP and other nucleotides do not interfere to any detectable extent. The results of this study indicate that cyclic AMP formation from radioactive ATP could be successfully assayed by the method described.

Based on the studies of adipose tissue adenylate cyclase (Kuo & DeRenzo, 1969), platelet adenylate cyclase has been studied by prelabelling these cells with adenine (Mills & Smith, 1971; Haslam & Rosson, 1975). In the prelabelling assay Mills & Smith (1971) incubated platelet-rich plasma with labelled adenine for 45–80 min at 37°C, whereas Haslam & Rosson (1975) prelabelled the platelets for 80–90 min. Using affinity elution chromatography we find that an incubation period of greater than 120 min is necessary for the labelling of cyclic AMP to reach a steady state (Fig. 2).

The affinity elution assay of cyclic AMP has been demonstrated to be highly specific (Sinha & Colman, 1977a). No detectable interference was caused by either cyclic GMP or various adenine nucleotides, including ATP. In the present study, the identity of the compound eluted from the QAE-cellulose by protein kinase was confirmed as cyclic AMP, by both t.l.c. and phosphodiesterase treatment of the eluted

material (Fig. 2). The assay described is simpler than any other conventional procedure and has the potential for use in other cell systems.

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