A MICRODETERMINATION OF CYCLIC AMP BASED UPON ACTIVATION OF A LIVER PROTEIN KINASE

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1. Introduction

Routine assay methods for adenosine 3′-5′-monophosphate (cyclic AMP) depend on conversion of phosphorylase b to a [1–3]. Recently, Walsh et al. [4] partially purified from rabbit muscle a cyclic AMP-dependent protein kinase, which is the initial link in the cascading series of protein phosphorylation reactions involved in this activation. Subsequently, a cyclic AMP-dependent protein kinase activity, measured by ^{32}P transfer to different proteins, has been found in several different tissues and species [5–8]. This report describes a partial purification of a cyclic AMP-dependent protein kinase from rat liver, and its use as a method for the assay of cyclic AMP in biological materials. The assay was based on the ability of cyclic AMP to increase the rate of histone phosphorylation when catalyzed with this liver protein kinase. Cyclic AMP was isolated from tissues, and an efficient purification of the extracts, removing many interfering substances was performed. The method proposed is simple, accurate, and allows the measure of cyclic AMP down to a level of 5×10^{-12} mole. Validity of the assay was checked on BHK 21 cells.

2. Materials and methods

2.1. *Preparation of liver protein kinase*

The partially purified enzyme was prepared essentially as described by Corbin and Krebs [6] for rat fat pads. Livers from 5 rats were pooled, and a 33% homogenate (w/v) in 0.25 M sucrose was prepared and centrifuged at 145,000 *g* for 2 hr. The supernatant was fractionated by addition of solid ammonium sulfate. The fraction which precipitated between 30 and 45% saturation, was dissolved in 0.02 M tris HCl buffer and pH 7.4, and dialyzed overnight in three changes of the same buffer. The specific activity of the enzyme preparation was about five-fold greater than that of the original homogenate. This preparation could be kept frozen in 1 ml fractions without appreciable loss of activity for several months.

2.2. *Standard assay*

The assay was slightly modified from a previously described method [5]. Assay reaction mixtures (1 ml) at pH 7.4 contained: tris-HCl buffer, 40 μmoles; $MgCl_2$, 50 μmoles; dithiothreitol, 1 μmole, $\gamma^{32}P$-labeled ATP, 0.08 μmole; histone, 1 mg; enzyme (about 1.5 mg protein); cyclic AMP or tissue extracts in varying amounts. The reaction was initiated by addition of the enzyme and the mixture was incubated at 37° for 5 min; the reaction was stopped by addition of 0.2 ml 0.01 M ATP, 0.2 ml serum albumin (12 mg/ml) and 1.4 ml 30% trichloroacetic acid (TCA). After centrifugation, the acid insoluble material was washed three times with 15% TCA and dissolved in 1 ml of 1.2 N NaOH at 80° for 45 min. Samples (0.2 ml) were added to 0.2 ml glacial acetic acid, 2 ml ethanol, 1 ml hyamine hydroxide, 10 ml of toluene scintillation fluid and counted in a Packard Tri-Carb liquid scintillation spectrometer. Each experimental series included an assay without histone.

2.3. *Tissue preparation*

Tissues were removed, crushed with a pre-cooled

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stainless steel tool and frozen by immersion in liquid nitrogen. Culture cells were collected by centrifugation and fozen in the same manner. Pre-weighed frozen tissue (or cells) was powdered in a cold stainless steel pulverizer. The powdered tissue was extracted and homogenized with 5 ml of 0.07 N perchloric acid per gram of tissue (or 10^8 cells). The homogenate was centrifuged at 85,000 g for 30 min. (Spinco rotor 30, polycarbonate tubes). The supernatant was collected and neutralized with 2 N KOH; then the $KClO_4$ precipitate was removed by centrifugation. A sample of the supernatant (5 ml) was concentrated at 50° *in vacuo* to a volume of 1 ml and an aliquot of 0.1 ml was spotted on 3 MM Whatman paper. Cyclic AMP was purified by descending chromatography for 16 hr, as described by Rabinowitz et al. [9]. The cyclic AMP zone was detected by UV absorption, since on each chromatogram a quantity (0.1 μmole) of cyclic AMP was run in parallel. The chromatogram was dried, and the cyclic AMP zone was cut out and eluted with 5 ml distilled water for 2–3 hr. The eluate was concentrated to 1 ml at 50° *in vacuo* before being assayed. In tissues which showed very low levels of cyclic AMP, as liver, the extract had to be concentrated two or three-fold further. In such cases, the resolution of the paper chromatography was not sufficient (several bases and nucleosides, plus a ninhydrin-positive material contaminated the cyclic AMP spot); therefore, electrophoresis in 0.05 M citrate buffer pH 3.5 for 90 min was performed prior to chromatography.

Protamine, serum albumin, casein, cyclic AMP and other adenylic derivates were purchased from Sigma Chemical Co; nucleotide 3′,5′-phosphodiesterase from beef heart and inosine 3′,5′-monophosphate (cyclic IMP), guanosine 3′,5′-monophosphate (cyclic GMP), deoxythymidine 3′,5′-monophosphate (cyclic dTMP), uridine 3′,5′-monophosphate (cyclic UMP), cytosine 3′,5′-monophosphate (cyclic CMP) were ordered from Boehringer Mannheim Corp. γ^{32}P-Labeled ATP was supplied by CEA France, Calf thymus histone was a gift from Laboratoires Choay.

3. Results

3.1. *Assay of cyclic AMP*

Fig. 1 shows the increased rate of histone phosphorylation, as a function of cyclic AMP concentration in the assay system. The amounts of ^{32}P transferred were corrected for phosphorylation of protein contaminants in the enzyme preparation, by being measured in absence of histone. This endogenous phosphorylation was unaffected by cyclic AMP. The half-maximal activation of histone phosphorylation was

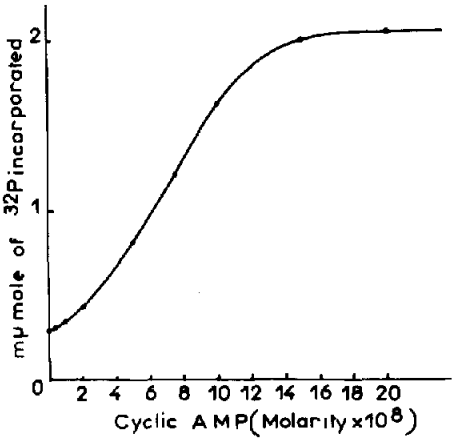

Fig. 1. Histone phosphorylation as a function of cyclic AMP concentration. A correction was applied for the amount of ^{32}P transferred to proteins from enzyme preparation (endogenous phosphorylation); in assay mixtures without protein substrate, it was evaluated as 0.16 mμmole either in the absence or presence of cyclic AMP.

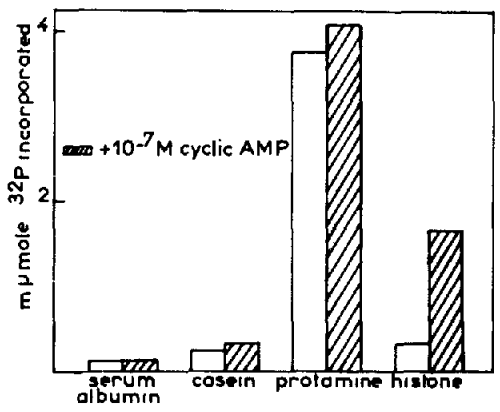

Fig. 2. A comparison of substrates for liver protein kinase. The substrate concentrations were 1 mg/ml. Other conditions were as described in the text. ^{32}P transferred to proteins was corrected for endogenous phosphorylation.

Table 1
Effect of various additions on ^{32}P incorporation in histone

Addition (5×10^{-4} M)	Cyclic AMP-independent phosphorylation		Cyclic AMP-dependent phosphorylation	
	^{32}P incorporated (mμmole)	% Inhibition	^{32}P incorporated (mμmole)	% Inhibition
None	0.29	–	1.35	–
Adenine	0.24	17	1.07	21
Hypoxanthine	0.23	21	1.16	13
Adenosine	0.26	10	1.13	16
Xanthosine	0.23	21	1.04	23
Inosine	0.18	38	0.89	34
5′-AMP	0.19	34	0.72	46
5′-ADP	0.06	79	0.35	74

Assay was performed under standard conditions, except for additions as indicated, in the absence (cyclic AMP-independent phosphorylation) or presence (cyclic AMP-dependent phosphorylation) of cyclic AMP (10^{-7} M). Figures were corrected for endogenous phosphorylation.

achieved at a concentration of cyclic AMP of approximately 7×10^{-8} M. The cyclic AMP standard curve is almost linear in the concentration range from 10^{-8} to 10^{-7} M. Reliability of the values was within 3%.

Histone was a better substrate than albumin, casein or protamine for liver cyclic AMP-dependent protein kinase as shown when these protein substrates were substituted for histone in the incubation system (fig. 2).

The effects of various additions to this standard system, such as bases, nucleosides or nucleotides, are illustrated in table 1. All compounds tested, at 5×10^{-4} M, inhibited ^{32}P transfer to histone. This inhibition was almost the same both in the absence or presence of cyclic AMP; therefore the different additions interfere only with phosphorylation reaction.

However, stimulation of the liver cyclic AMP-dependent protein kinase did not show an absolute specificity. Another 3′,5′-nucleotide, inosine 3′,5′-monophosphate, was active: half-maximal stimulation by cyclic IMP was obtained at a concentration of about 6×10^{-7} M; at the same concentration, cyclic IMP was approximately 10% as effective as cyclic AMP. Adenosine 2′,3′-monophosphate and other 3′,5′-nucleotides did not stimulate the enzyme significantly; however, slight contamination by cyclic AMP could not be ruled out (table 2).

3.2. *Measurement of cyclic AMP in biological materials*

This assay method has been applied to different materials (tissues, culture cells, culture medium, serum). Tests of validity developed in this report were applied to BHK 21 cells cultivated in suspension [10].

The assay method developed is specific for cyclic AMP. Indeed, in the solvent system used, the R_f for cyclic AMP, cyclic dTMP, cyclic CMP, cyclic UMP, cyclic IMP, cyclic GMP were respectively: 0.43, 0.42, 0.37, 0.29, 0.26, 0.16. Thus, the only other cyclic

Table 2
Relative ability of cyclic nucleotides to stimulate the liver protein kinase activity

Cyclic nucleotide (10^{-7} M)	^{32}P incorporated (mμmole)	% Activation
None	0.29	–
Adenosine 3′,5′-monophosphate	1.64	465
Inosine 3′,5′-monophosphate	0.44	52
Guanosine 3′,5′-monophosphate	0.30	3
Cytosine 3′,5′-monophosphate	0.29	0
Thymidine 3′,5′-monophosphate	0.27	0
Uridine 3′,5-monophosphate	0.27	0
Adenosine 2′,3′-monophosphate	0.28	0

Assay conditions were as described in the text, except for the cyclic nucleotides (10^{-7} M) present. Figures were corrected for endogenous phosphorylation.

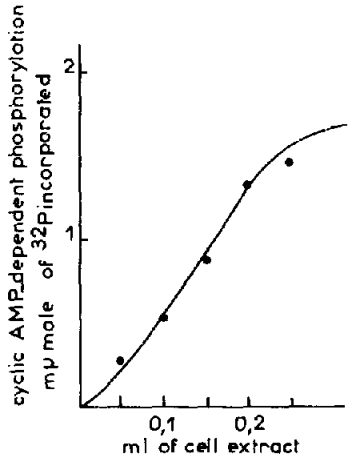

Fig. 3. Increase of histone phosphorylation as a function of the volume added of BHK 21 cell extracts. ^{32}P transferred to histone in absence of cyclic AMP was subtracted.

nucleotide besides cyclic AMP, binding liver protein kinase, was cyclic IMP, which can easily be removed by the purification procedure.

The relationship between the increased rate of ^{32}P transfer to histone and the volume of cell extract added to the assay system is shown in fig. 3. Extract dilutions were chosen to obtain a stimulation of histone phosphorylation in the range of 0.2 to 1.5 mμmole of ^{32}P incorporated under standard conditions. Similarity between this curve and the standard curve indicated that there were no substances interfering with the reaction.

Addition of known amounts of cyclic AMP marker (5×10^{-8} or 10^{-7} M) in 0.07 N perchloric acid (first step of tissue extraction) and measurement of protein kinase activation, allowed the assessment of the recovery of cyclic AMP, plus the extent of inhibition by cell extract contaminants. Results ranged from 80 to 95%, which is a high yield and evidence for negligible contamination.

To further assess the validity of the assay, a test involving the breakdown of cyclic AMP in cell extracts by nucleotide 3′,5′-phosphodiesterase action prior to assay, was applied by the modified method (manuscript in preparation) of Butcher and Sutherland [11]. The cell extracts incubated 30 min at 37° lost 85 to 90% of their ability to stimulate protein kinase. It must be pointed out that in this assay of cyclic AMP, this test is available, although generally it is not. Two enzymic activities in the commercial phosphodiesterase preparation were found (unpublished data): one hydrolyzing cyclic AMP and cyclic IMP, another hydrolyzing cyclic GMP. This observation confirmed the data of Brooker et al. [12] on a cyclic phosphodiesterase brain preparation. In the assay method developed, cyclic GMP did not activate liver protein kinase and paper chromatography allowed a good separation of cyclic AMP from cyclic IMP. Thus, in this case, nucleotide 3′,5′-phosphodiesterase attack was an available test.

Consequently, the assay method for cyclic AMP proposed is specific; moreover, it is as sensitive and accurate as methods based upon conversion of phosphorylase b to a. Also it is simple because it involves an enzyme and substrates that are easy to prepare, and depends on the initial action of cyclic AMP to stimulate phosphorylation reactions.

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