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Short Communication

Basal and stimulated cyclic adenosine 3′,5′-monophosphate production in rat adenopituitary homogenates: direct determination by high-performance liquid chromatography

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ABSTRACT

A sensitive and reliable procedure to quantify cAMP in biological samples, without involving radioisotopes, is described. After incubation, the assay mixture was boiled for 4 min, centrifuged, filtered and directly injected onto a μBondapak C_{18} column (10 μm particle size; 150 mm × 3.9 mm I.D.). cAMP was resolved within 40 min in isocratic mode using a mobile phase composed of 96% of 0.01 *M* ammonium acetate, pH 6.6 and 4% of acetonitrile–water (70:30, v/v) at a flow-rate of 0.5 ml/min. Detection was monitored at 254 nm and quantification was achieved by peak surface integration. The lowest detection limit was 1.6 pmol (signal-to-noise ratio = 2.5). The potential of this assay to perform structure–activity of bioactive peptides is illustrated with growth hormone-releasing factor in rat adenopituitary homogenates.

INTRODUCTION

Cyclic adenosine 3′,5′-monophosphate (cAMP) is an important second messenger that mediates cellular responses in G-protein-coupled receptor systems [1]. The quantification of cAMP requires a very sensitive and specific method because of its low concentration in biological samples and the presence of numerous side-products coming from the adenylate cyclase assay [2]. Up to now, determination of basal and stimulated cAMP levels in tissue preparations has mainly been accomplished by radioactive detection. Either *de novo* formed [^{32}P]cAMP is quantified after its separation on an individual series of ion-exchange and neutral alumina open columns [2] or directly measured using ^{3}H-based radioimmunoassays (RIA) [3]. Although the second method is less time-consuming than the first, both have the disadvantage of requiring the use of radiolabelled compounds.

Reversed-phase high-performance liquid chromatography (HPLC) with UV [4,5] or fluorometric [6] detection has been proposed as a rapid and sensitive alternative to measure cAMP in biolog-

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ical samples. In most of these reports, large quantities of tissue proteins were involved, limiting their application to extensive studies on peptide structure–activity relationship.

In the present investigation, we describe a reversed-phase HPLC method using UV detection for direct determination of picomole levels of cAMP. The applicability of the method is demonstrated in rat adenopituitary homogenates with two growth hormone-releasing factor (GRF) receptor agonists and one antagonist and with forskolin, an agent that directly stimulates adenylate cyclase.

EXPERIMENTAL

Materials

The synthetic peptides used in this study, human (h) GRF(1–29)NH_2, [D-Tyr^1]hGRF(1–29)-NH_2 and Nα-Ac-[His^1,D-Arg^2,Ala^{15}] rat (r) GRF(1–29)NH_2, were synthesized, purified and characterized according to Gaudreau *et al.* [79]. Accusolv-grade acetonitrile was purchased from Anachemia Canada (Lachine, Canada) and ammonium acetate from Baker (Phillipsburg, NJ, USA). Ethylenediaminetetraacetic acid (EDTA), ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA), forskolin, magnesium chloride and sucrose were obtained from Sigma (St. Louis, MO, USA). Adenine, inosine, adenosine, tris(hydroxymethyl)aminomethane and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Aldrich (Milwaukee, WI, USA). cAMP, adenosine 5′-monophosphate (AMP), adenosine 5′-diphosphate (ADP), adenosine 5′-triphosphate (ATP), guanosine 5′-triphosphate (GTP), myokinase, creatine phosphate (CP) and creatine kinase (CK) were purchased from Boehringer Mannheim (Laval, Canada).

Preparation of adenopituitary homogenates

Male Sprague–Dawley rats (200–225 g) were purchased from Charles River Canada (St. Constant, Canada). Anterior pituitaries (pit.) were dissected out immediately after decapitation, rinsed and homogenized for 8 s in ice-cold 20 m*M* Tris–HCl buffer (pH 7.5) containing 2 m*M* $MgCl_2$ and 250 m*M* sucrose (1 pit. per 0.5 ml) using a micro ultrasonic cell disrupter (Kontes, Vineland, NJ, USA). This homogenate was used within 10 min for the adenylate cyclase assay.

Adenylate cyclase assay

The adenylate cyclase assay was performed according to the procedures of Robberecht *et al.* [8] and Anand-Srivastava *et al.* [9] with minor modifications. Samples of anterior pituitary homogenates (30–40 μg of protein) were incubated in 1.5-ml Eppendorf tubes with 30 m*M* Tris–HCl, 5 m*M* $MgCl_2$, 0.5 m*M* EGTA (assay buffer), containing 0.5 m*M* ATP, 1 m*M* IBMX and an ATP-regenerating system consisting of 2 m*M* CP, 0.1 mg/ml CK and 0.1 mg/ml myokinase in a final volume of 120 μl. IBMX (50 m*M*) and forskolin (3 m*M*) were solubilized in methanol and ethanol, respectively. When peptides (40 μl) were tested, 10 μ*M* GTP was added to the incubation medium. GRFs (300 μ*M*) were solubilized in 0.1 *M* HCl–0.1 *M* NaOH (51:49) and further diluted in the assay buffer.

Incubation was initiated by adding 20 μl of the homogenate at 10-s intervals to the reaction mixture which had previously been equilibrated at 37°C for 2 min. The reactions were carried out for 8 min at 37°C and stopped sequentially by heating the samples in boiling water for 4 min. Samples were then centrifuged (12 000 *g*, 5 min, 4°C). The supernatants were filtered through Millex HV_4 filters (0.45 μm; Millipore, Mississauga, Canada), transferred to HPLC autosampler vials and analysed. Proteins concentration was determined by the Bradford method [10] using bovine serum albumin as standard.

Quantification of cAMP by HPLC

cAMP was chromatographed on a μBondapak C_{18} column (10-μm particles; 150 mm × 3.9 mm I.D.) (Waters, Mississauga, Canada). Samples (40 μl) were injected onto the column using a Wisp 712 automated sample injector (Waters). The flow-rate was maintained at 0.5 ml/min using a Model 510 solvent delivery system (Waters). The mobile phase was composed of 96% 0.01 *M* ammonium acetate pH 6.6 and 4% acetonitrile–

water (70:30, v/v). All the HPLC solvents were filtered (HVLP and HAWP filters, 0.45 μm; Millipore) and degassed. Each run was performed in the isocratic mode for 40 min at room temperature. Peptides such as GRFs were retained on the column in these conditions. Therefore, after each series of twelve injections, the column was washed out with 30 ml of acetonitrile at a flow-rate of 1.5 ml/min.

Detection of cAMP was performed at 254 nm (sensitivity: 0.01 a.u.f.s.) using a Model 440 UV detector (Waters) equipped with a 1 cm path length cell. cAMP was quantified by peak surface integration in comparison with an external standard of cAMP, using a Model SP4270 integrator (Spectra-Physics, San Jose, CA, USA) (chart speed: 0.25 cm/min; attenuation: 2).

Standard solutions of cAMP (100 nmol) were prepared in the assay buffer and kept frozen in aliquots at −20°C. Prior to analyses, they were diluted in the incubation medium to a concentration of 10 pmol per 40-μl injection volume. An external standard was run every five samples.

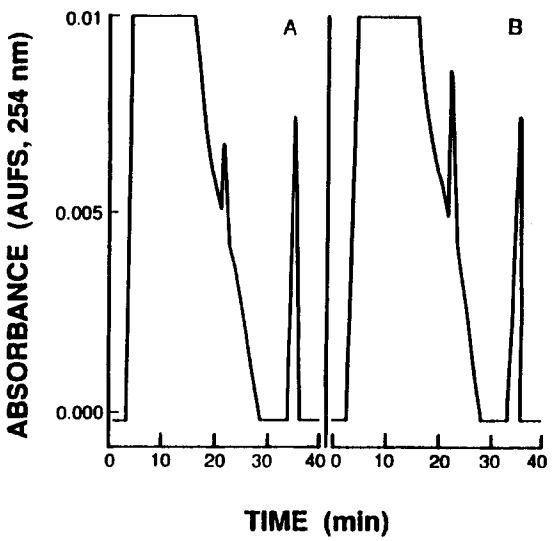

Fig. 1. Chromatographic profiles of cAMP in the assay mixture. (A) 10 pmol of cAMP standard alone or (B) in admixture with *de novo* cAMP produced by 10 n*M* hGRF(1–29)NH_2. The solvent system was composed of 96% 0.01 *M* ammonium acetate pH 6.6 and 4% acetonitrile–water (70:30, v/v). Elution was achieved on a μBondapak C_{18} column in the isocratic mode at a flow-rate of 0.5 ml/min. Retention time of cAMP was 22.3 min.

RESULTS AND DISCUSSION

Evaluation of the method for cAMP quantification

Before undertaking quantification of cAMP, the chromatographic conditions necessary to achieve optimal separation were determined in our assay conditions. Ammonium acetate and acetonitrile were used as mobile phases to minimize HPLC maintenance associated with utilization of potassium or tetrabutylammonium phosphate buffers [4,5]. It was found that 96% 0.01 *M* aqueous ammonium acetate pH 6.6 and a 4% mixture of 70:30 (v/v) acetonitrile–water was the best combination for separating cAMP from the side-products of the reaction mixture. cAMP was well resolved in this solvent system in the isocratic mode. As shown in Fig. 1A, the retention time of cAMP was 22.3 ± 0.2 min. The use of isocratic elution was advantageous at high sensitivity to reduce stabilization time of the UV detector.

To ensure that our HPLC protocol effectively resolved cAMP from other nucleotides and compounds present in the assay medium, standards of various adenine nucleotides and other related molecules were analysed (12.5 pmol per 25 μl). AMP, ADP and ATP eluted within the first 6 min, inosine, adenine and adenosine eluted at 14.0, 16.9 and 36.7 min, respectively. Furthermore, when the assay medium was injected alone (40 μl), no peak was observed at 22.3 min. Calibration curves for cAMP were linear between 1.6 and 100 pmol and the intra- and inter-assay variation was 4 and 10% for 10 pmol, respectively. The identify of the *de novo* cAMP peak was further ascertained by co-chromatography with a cAMP standard, as shown in Fig. 1B.

Our assay procedure was adapted to be compatible with HPLC measurements. Thus, production of cAMP was stopped by boiling samples instead of adding sodium dodecyl sulfate (SDS) [8], acid [11] or alcohol [12] in the reaction mixture. It was chosen to avoid sample dilution (acid, alcohol) or HPLC column damage. In addition, 1 m*M* IBMX was used to inhibit phosphodiesterase activity [11]. Under these conditions, GRF-induced (10 n*M*) formation of cAMP

TABLE I

ADENYLATE CYCLASE ACTIVITY IN RAT ADENOPITUITARY HOMOGENATES INDUCED BY GTP, FORSKOLIN, hGRF(1–29)NH_2, [D-TYR^1]hGRF(1–29)NH_2 AND Nα-Ac-[HIS^1,D-ARG^2,ALA^{15}]rGRF(1–29)NH_2

The effect of peptides was evaluated in the presence of 10 μM GTP. Results are expressed after subtraction of the unstimulated value and represent the mean ± S.D.

Compound	Concentration	cAMP (pmol/min/mg protein)	n
GTP	10 μM	100 ± 9	3
Forskolin	10 μM	467 ± 70	3
hGRF(1–29)NH_2	1 nM	94 ± 22	4
	10 nM	119 ± 22	4
	100 nM	186 ± 29	4
	1 μM	264 ± 22	4
[D-Tyr^1]hGRF(1–29)NH_2	1 nM	40 ± 17	2
	10 nM	79 ± 11	2
	100 nM	168 ± 12	2
	1 μM	225 ± 39	2
Nα-Ac-[His^1,D-Arg^2,Ala^{15}]rGRF(1–29)NH_2	1 μM	14 ± 13	3

was linear over time for at least 10 min at 37°C, as previously documented [2,8] and stable for at least 24 h at room temperature (data not shown).

With the HPLC system used, the lowest limit of detection was 1.6 pmol, with a corresponding signal-to-noise (peak height/baseline height) ratio of 2.5; the signal-to-noise ratio increased to 5 for 3.1 pmol cAMP. This is less sensitive than for fluorometric detection (0.1 pmol [6]) but with the more sensitive instrumentation now available it should be possible to improve the detection limit to 0.05–0.1 pmol. Moreover, UV detection simplifies the procedure by circumventing the need of cAMP derivatization as for fluorometric de-

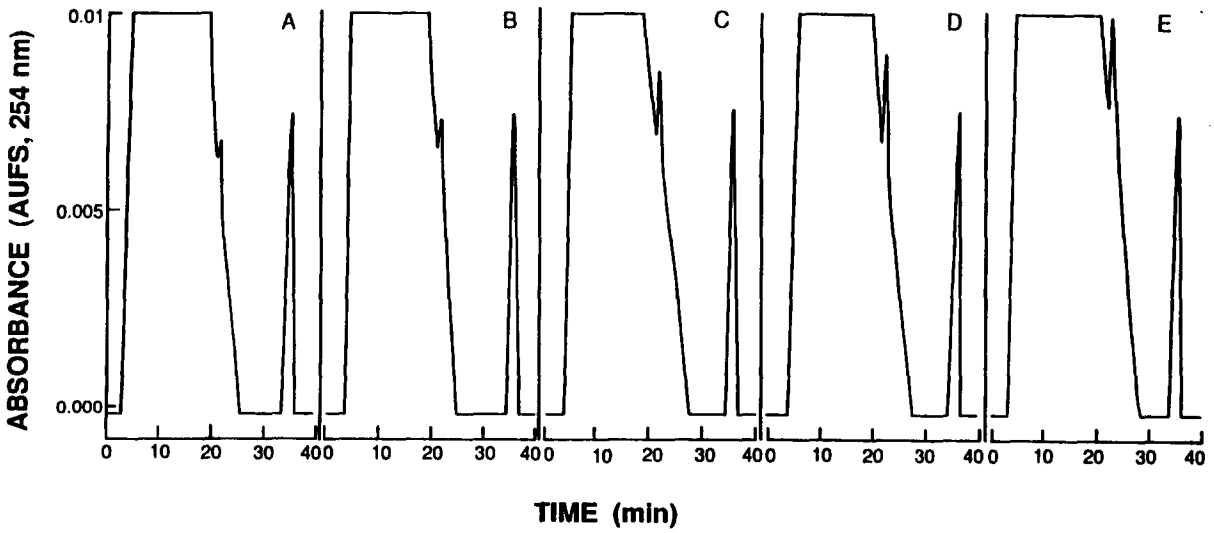

Fig. 2. Chromatogrpahic profiles of hGRF(1–29)NH_2-induced cAMP production. Panels A, B, C, D and E represent basal and 1 nM, 10 nM, 100 nM and 1 μM hGRF(1–29)NH_2-induced cAMP production, respectively. The solvent system was composed of 96% 0.01 M ammonium acetate pH 6.6 and 4% acetonitrile–water (70:30, v/v). Elution was achieved on a μBondapak C_{18} column in the isocratic mode at a flow-rate of 0.5 ml/min. Retention time of cAMP was 22.3 min.

tection [6], thus minimizing losses. Altogether, our approach provides an efficient and reliable method of quantifing picomole amounts of cAMP that can be applied in rat adenopituitary homogenates to perform GRF structure–activity studies.

Application in rat adenopituitary homogenates

It has been recently demonstrated that the pituitary GRF receptor is a member of the G-protein-coupled receptor family and its activation by GRF agonistis promotes cAMP synthesis [13,14]. As shown in Table I, GTP (10 μM), forskolin (10 μM) and hGRF(1–29)NH_2 (1 nM–1 μM) induced a 1.5- to 2-fold higher cAMP production than previously reported [8]. This is likely due to our direct HPLC method of quantification. Both GRF agonists, hGRF(1–29)NH_2 (Table I and Fig. 2) and [D-Tyr^1]hGRF(1–29)NH_2 (Table I) induced a concentration-dependent production of cAMP. Their respective EC_{50} (concentration of peptide inducing 50% of maximal cAMP production with 1 μM hGRF(1–29)NH_2) were 10.4 and 38.0 nM. The relative potency of [D-Tyr^1]hGRF(1–29)NH_2 compared to hGRF-(1–29)NH_2 was similar in our assay to that of quantification of *in vitro* growth hormone production [15]. As expected, the antagonist, Nα-Ac-[His^1,D-Arg^2,Ala^{15}]rGRF(1–29)NH_2 (1 μM), was inactive [16].

CONCLUSION

The procedures described in this paper offer many advantages over radioisotopic existing methods [2,3,8]. These advantages are mainly due to the potential for HPLC to directly quantify cAMP. The method is sensitive enough to detect basal and stimulated cAMP in small amounts of tissue homogenates, for example, 1/20 (30 μg) rat adenopituitary, and is a valuable tool for the assessment of structure–activity relationships of bioactive peptides.

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