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RAPID AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF CYCLIC ADENOSINE 3′,5′-MONOPHOSPHATE SYNTHESIS IN BRAIN TISSUES

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SUMMARY

A rapid and sensitive automated system for measuring cyclic adenosine 3′,5′-monophosphate (cAMP) synthesized from either radiolabelled adenine or adenosine 5′-triphosphate (ATP) in intact and broken cell tissue preparations, respectively, is described. After incubation with radiolabelled precursor, tissue samples are deproteinized and then injected directly onto a reversed-phase high-performance liquid chromatographic column. The column effluent fraction which contains cAMP is collected into scintillation vials and assayed for tritium by liquid scintillation spectrometry. Since the high-performance liquid chromatographic and fraction collection procedures are automated, over fifty samples can be analyzed in duplicate in a single day. The utility of this assay is illustrated by investigations of the effects of β-adrenergic receptor stimulation on cAMP synthesis in tissue slices prepared from rat cerebral cortex and dopamine on cAMP synthesis in striatal membrane preparations.

INTRODUCTION

It is well established that cyclic adenosine 3′,5′-monophosphate (cAMP) is a second messenger which mediates many cellular responses to receptor stimulation [1, 2]. Changes in cAMP synthesis in response to various stimuli have been measured, using radioisotopic methods, in both intact and broken cell preparations. These methods are based upon the measurement of cAMP formation from either radiolabelled adenine or adenosine 5′-triphosphate (ATP) [3—10]. The use of high specific activity [^{32}P]ATP permits the sensitive analysis of cAMP synthesis, but its application is limited to studies with broken cell preparations, since ATP does not readily cross cell membranes. The use of radiolabelled adenine, which can be taken up by cells and converted to ATP,

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provides a useful alternative for measuring cAMP synthesis in intact cells or tissue slices [5—9]. Moreover, assays utilizing adenine can be applied to examine the effects of drugs on cAMP synthesis in ex vivo studies, whereas in methods which use ATP, drugs administered in vivo are washed out or greatly diluted when cell membrane fractions are prepared.

Typically, radioisotopic assays for cAMP synthesis have used one or more ion-exchange columns or other time-consuming chromatographic steps to separate cAMP from radiolabelled precursors and contaminants. Such tedious procedures are subject to errors if, for example, the elution profile of columns varies. Recently, Martinez-Valdez et al. [11] reported a high-performance liquid chromatographic (HPLC) method for separating purine and pyrimidine nucleotides. Using that chromatographic system as a starting point, we have modified it and developed a rapid system for separating cAMP from adenine, ATP, and other adenine-derived compounds, and we have automated and applied this system to the measurement of cAMP synthesis in brain tissue slices and broken cell preparations. While this manuscript was in preparation, Schulz and Mailman [10] presented an excellent method for measuring cAMP synthesis from [^{32}P]ATP in broken cell preparations. Their procedure also utilizes HPLC to purify cAMP. However, data were not presented on the resolution of cAMP from adenosine. Such resolution is necessary if cAMP synthesis is measured either in tissue slices using [^{3}H]adenine or in membrane preparations using [^{3}H]ATP. Using modifications of previously described incubation conditions for the measurement of adenylate cyclase [4, 5, 8, 9], both of these applications are described below.

EXPERIMENTAL

Measurement of cAMP synthesized from adenine in tissue slices

Male Sprague—Dawley rats (Hilltop Labs., Scottdale, PA, U.S.A.) weighing 170—230 g, are decapitated and tissues (e.g., occipital cortex) are immediately dissected out on an ice-cooled plate. Tissue slices (300 × 300 μm) are prepared using a McIlwain tissue chopper (Brinkman Instruments, Westbury, NY, U.S.A.). Then, the tissue slices are suspended in approximately 20 vols. (w/v) ice cold Krebs—Ringer bicarbonate buffer, pH 7.2 which is equilibrated with oxygen—carbon dioxide (95:5). After centrifugation (500 *g* for 1 min), the tissue pellet is resuspended in 20 vols. (w/v) of the Krebs—Ringer buffer.

[^{3}H]Adenine is mixed with the tissue suspension (5 μCi/ml; 287 n*M* final concentration in most experiments). Next, 1.2 ml of the suspension are incubated at 37°C in a shaking water bath under an oxygen—carbon dioxide (95:5) atmosphere. After a 45-min pre-incubation at 37°C to label ATP pools, samples are incubated with isoproterenol or other agonists in 10 μl of 0.1% (w/v) ascorbic acid (see Results). At the end of the incubation, 600 μl of 0.3 *M* sodium hydroxide and 10 μl water containing 50 μg cAMP are added to each sample and vortexed. After allowing samples to stand at room temperature (22°C) for 30 min, 100 μl are removed for protein analysis and 1.5 ml are transferred to 15-ml plastic centrifuge tubes which contain 0.5 ml of 0.15 *M* zinc sulfate. The samples are centrifuged at 10 000 *g* for 20 min at 4°C and aliquots of the supernatant (about 1.5 ml) are transferred to the sample vials

used by the HPLC automatic sample injector (see below). Care should be taken not to disturb the precipitate. Sample blanks are prepared using the above procedures, except that the tissue is boiled for 5 min and cooled to 4°C prior to the addition of [^{3}H]adenine.

Measurement of cAMP synthesized from ATP

Male Sprague—Dawley rats, weighing 170—230 g, are decapitated and tissues (e.g, striata) are rapidly dissected out on ice. Tissues are then homogenized with a ground glass pestle in 20 vols. (w/v) ice cold saline containing 0.5 m*M* ethyleneglycol-bis(β-aminoethyl ether)-N,N′-tetraacetic acid (EGTA) and buffered with 25 m*M* Tris · HCl, pH 7.5. After centrifugation at 300 *g* for 30 sec to remove debris, 100 μl supernatant are transferred to ice cold tubes which contain 70 μl reaction mixture (20 m*M* creatine phosphate, 5 U rabbit muscle creatine phosphate kinase, 1.5 m*M* 3-isobutyl-1-methylxanthine, and 2 m*M* cAMP in 100 m*M* Tris maleate buffer, pH 7.5) and 10 μl each of receptor agonists and antagonists or their diluent (generally 0.1% ascorbic acid). Sample blanks are prepared by boiling the sample homogenate for 5 min and then cooling the samples to 4°C prior to the above additions. The enzyme reaction is started by adding, at 10-sec intervals, 10 μl of a solution which contains 50 m*M* ATP (approximately 0.5 μCi [^{32}P]ATP or 1 μCi [^{3}H]ATP) and 200 m*M* magnesium chloride and immediately placing the samples in a shaking water bath at 37°C. After a 10-min incubation at 37°C, the reaction is stopped, at 10-sec intervals, with 0.2 ml of 0.3 *M* sodium hydroxide and the samples are vortexed. Then, 0.2 ml of 0.15 *M* zinc sulfate is added to each sample. After centrifugation at 5000 *g* for 50 min, 200 μl supernatant are transferred to HPLC autosampler vials. Proteins are determined using aliquots of the original homogenate.

Purification of cAMP by HPLC

cAMP is separated from other radiolabelled adenine derivatives on a 5-μm, 15 cm × 4.6 mm Ultrasphere ODS® reversed-phase column (Rainin Instruments, Woburn, MA, U.S.A.). A 5-μm, 4.0 cm × 4.6 mm precolumn is inserted between the analytical column and the sample injector to protect the analytical column from sample particulates. Samples (80 μl) are injected onto the columns using WISP 710B automatic sample injector (Waters Assoc., Milford, MA, U.S.A.). Mobile phase flow-rate is maintained at 1 ml/min using a Model 112 solvent delivery module (Beckman Instruments, Berkeley, CA, U.S.A.). The HPLC mobile phase consists of 0.2 *M* ammonium phosphate, pH 3.0—methanol (100:1, v/v).

The position of the HPLC column effluent fraction containing cAMP is monitored with a UV detector (254 nm; Model 153, Beckman Instruments). The output of the UV detector is connected to a peak separator (Model 2150, ISCO, Lincoln, NB, U.S.Al) which then triggers a programmable Foxy® fraction collector (ISCO). The fraction collector is equipped with a diverter valve which directs the cAMP fraction (1.5 ml) into 7-ml polypropylene scintillation vials and shunts the remaining effluent into a waste receptacle. Liquiscint® scintillation cocktail (5 ml) from National Diagnostics (Somerville, NJ, U.S.A.) is added to each vial and radioactivity is measured by liquid scintillation spectrometry. Counting efficiency is determined using ^3H_2O.

The output of the UV detector is also connected to an integrator, which is equipped with a printer/plotter (Model 3390A, Hewlett-Packard, Avondale, PA, U.S.A.). The integrator reports are used to verify the position of the cAMP peaks and to check sample recoveries (as measured by cAMP peak area owing to added cAMP carrier). Prior to each assay, 100 μl water containing 10 μg each of adenine, adenosine, ATP, and cAMP are injected into the HPLC system. This is carried out as a quality control to verify that the chromatographic system adequately resolves these compounds. Typically, the system, as described, can be used nearly continuously for many months without noticeable decreases in performance. If a decrease in HPLC column performance does occur, column performance can generally be returned to normal by washing the column according to the manufacturer's instructions.

Miscellaneous

2,3[^{3}H]Adenine (17.4 Ci/mmol), 2,8,5'[^{3}H]ATP (46.2 Ci/mmol), and [^{32}P]ATP (600 Ci/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). Adenine, adenosine, and isobutyl methylxanthine were purchased from Sigma (St. Louis, MO, U.S.A.); creatine phosphate and rabbit muscle creatine phosphate kinase were purchased from Calbiochem (La Jolla, CA, U.S.A.). All other reagents are of the highest grade available. Proteins are assayed by the method of Lowry et al. [12]. cAMP synthesis rates were calculated by dividing the dpm present in the cAMP fraction by the specific activity of the added precursor (i.e. [^{3}H]adenine or [^{3}H]ATP) and are expressed as the molar concentration of cAMP formed per mg protein in the original sample aliquot per min of incubation at 37°C.

RESULTS

cAMP purification

Before undertaking measurements of cAMP synthesis, the conditions necessary for the separation of adenine and other adenine-derived compounds were determined. Modification of the chromatographic system of Martinez-Valdez et al. [11] provided an efficient and reliable separation of cAMP from adenine, adenosine, AMP, ADP, and ATP. Fig. 1 shows the elution profile obtained following the injection of a tissue sample incubated with [^{3}H]adenine. The cAMP containing fraction (peak 4) is well separated from the fractions containing ADP and ATP (peak 1), AMP and adenine (peak 2), and adenosine (peak 3). Similar elution profiles were observed when samples were incubated with [^{3}H]ATP, except that the adenosine fraction was reduced. When [^{32}P]ATP was used, no radioactivity was found in the adenosine fraction. Varying the composition of the HPLC mobile phase produced equivalent shifts in the retention times of the cAMP peak from tissue samples and authentic [^{3}H]cAMP, indicating that the radioactivity in the cAMP peak from tissue samples was attributable to cAMP. Pharmacological studies, described below, also confirm the identity of the cAMP peak from tissue samples.

Sodium hydroxide was used to stop cAMP synthesis as well as to solubilize tissues for protein analysis. Tissue solubilization with detergents, such as sodium dodecyl sulfate (SDS), should be avoided, since they can inactivate the HPLC

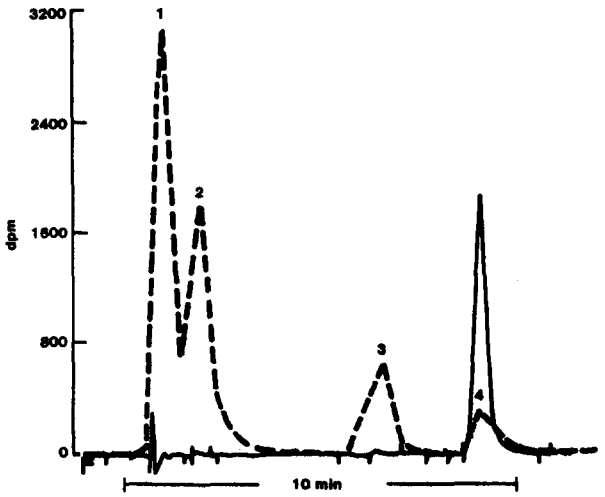

Fig. 1. Elution profile of radioactivity from HPLC column, after injection of deproteinized rat cortical tissue slices that had been incubated at 37°C for 60 min with [^{3}H]adenine. Peaks: 1 = ADP and ATP; 2 = AMP and adenine; 3 = adenosine; 4 = cAMP. Solid line is the UV tracing owing to the injection of unlabelled cAMP. See text for details.

column. After protein denaturation with sodium hydroxide, adenine and adenine-derived compounds other than cAMP were precipitated using zinc sulfate. However, adenosine was not precipitated under these conditions (data not shown). Samples deproteinized as described under Experimental were stable for at least 24 h at room temperature (22°C).

Isoproterenol-stimulated cAMP synthesis

The effects of isoproterenol, a β-adrenergic receptor agonist which stimulates cAMP synthesis [8], were examined in tissue slices prepared from rat occipital

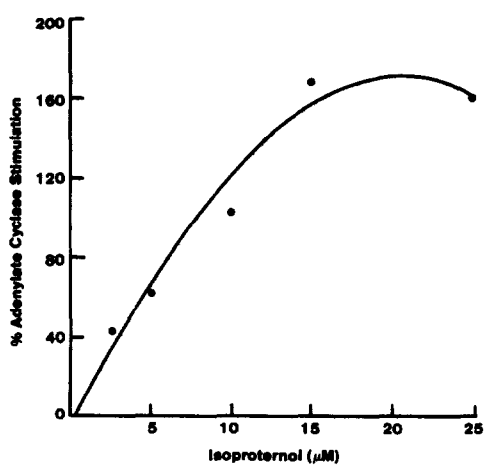

Fig. 2. Stimulation of cAMP synthesis in rat occipital cortical slices by isoproterenol. Slices were pre-incubated with [^{3}H]adenine for 45 min to label ATP stores, and then with isoproterenol for 15 min as described in Experimental. Basal cAMP rates were 27.9 pmol/mg protein per min.

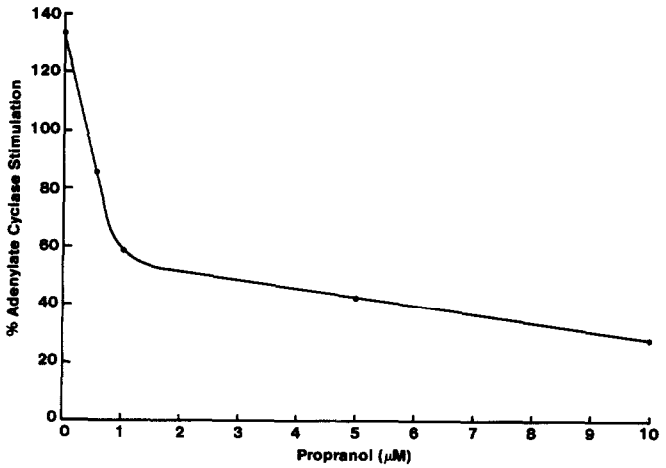

Fig. 3. Effect of propranolol on the response to isoproterenol. Occipital cortical slices were pre-incubated with [^{3}H]adenine for 45 min and then with various concentrations of propranolol for 10 min, followed by 25 μM isoproterenol for 15 min. Basal cAMP synthesis rates were 25.0 pmol/mg protein per min.

cortex. Slices were first pre-incubated for 45 min with [^{3}H]adenine and then with 0—25 μM isoproterenol. In agreement with others [8], using similar incubation conditions, isoproterenol increased tissue cAMP concentrations (Fig. 2) and this apparent stimulation of adenylate cyclase was attenuated in a dose-dependent manner by propranolol, a β-adrenergic receptor antagonist (Fig. 3).

Dopamine-stimulated cAMP synthesis

It is well known that dopamine (DA) receptor stimulation can promote cAMP synthesis [8, 10, 13, 14]. Fig. 4 illustrates the concentration-dependent stimulation of cAMP synthesis in rat striatal membrane preparations incubated with [^{32}P]ATP. Haloperidol, a DA receptor blocker, antagonized the

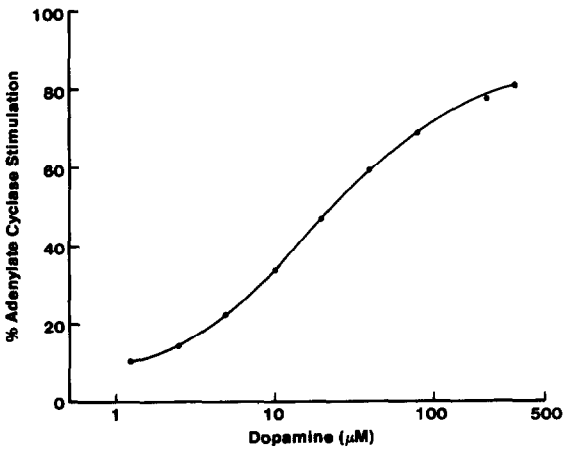

Fig. 4. Dopamine-stimulated cAMP synthesis in a rat striatal membrane preparation. Samples were incubated with [^{32}P]ATP and varying concentrations of dopamine, as described in Experimental. Basal cAMP synthesis rates were 283 fmol/mg protein per min.

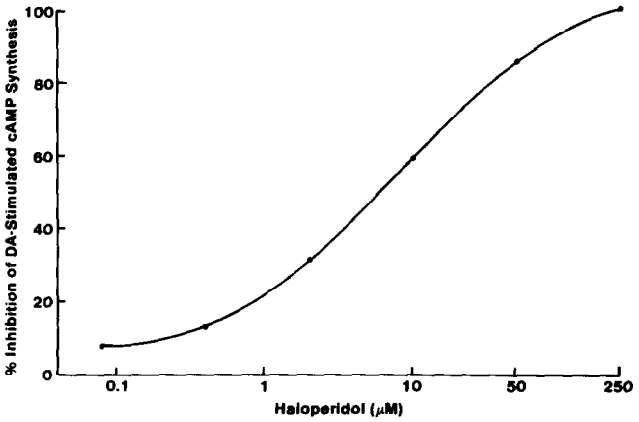

Fig. 5. Inhibition by haloperidol of dopamine-stimulated cAMP synthesis. Rat striatal membranes were incubated [^{3}H]ATP, 100 μM dopamine, and varying concentrations of haloperidol, as described in the text. Basal cAMP synthesis rates were 239.6 fmol/mg protein per min.

TABLE I

INTRA- AND INTER-ASSAY RELIABILITY

The ability of 25 μM isoproterenol to stimulate [^{3}H]cAMP formation from [^{3}H]adenine in occipital cortical tissue slices and the ability of 100 μM dopamine to stimulate [^{3}H]cAMP synthesis from [^{3}H]ATP in striatal membrane preparations were measured as described in Experimental.

Precursor	Single assay		Multiple assays	
	Percentage stimulation (mean ± S.E.M.)	Coefficient of variation (%)	Percentage stimulation (mean ± S.E.M.)	Coefficient of variation (%)
[^{3}H]Adenine	134 ± 2 (6)*	4.8	132 ± 2 (7)**	3.1
[^{3}H]ATP	31 ± 1 (8)*	3.2	44 ± 1 (28)**	8.1

*Number of determinations.
**Number of assays on separate days, triplicate determinations.

stimulation produced by 100 μM DA in a concentration-dependent manner when striatal membranes were incubated with [^{3}H]ATP (Fig. 5).

Assay reliability

The reliability of these assay procedures was investigated by determining the intra- and inter-assay coefficients of variation for tissue slices incubated in the presence and absence of isoproterenol and for membrane preparations incubated in the presence and absence of DA (Table I). The relatively low coefficients of variation indicate the high reliability of this assay.

DISCUSSION

The procedures described in this report offer several advantages over existing methods. These advantages are derived primarily from the HPLC system used

to separate radiolabelled cAMP from radioactive contaminants present in tissue extracts. Rather than using tedious and time-consuming extraction procedures, operator time is reduced through the use of an automated HPLC system. Likewise, the high efficiency and reproducibility of the HPLC purification procedures permits the reliable analysis of cAMP synthesis in small amounts of tissue (Table I). The extremely high efficiency of HPLC procedures relative to conventional column chromatography and other extraction procedures also ensures that the purification procedures are specific for cAMP (Fig. 1).

Barium hydroxide and zinc sulfate have been used to remove adenine and non-cyclic adenine nucleotides in several adenyl cyclase assays [3]. Sodium hydroxide is used in this assay, instead of barium hydroxide, to inactivate tissue enzymes. Barium hydroxide, under our assay conditions, produced some loss of cAMP (unpublished observations). In addition, the use of sodium hydroxide permits the solubilization of tissue slices for protein analysis. Although this assay is already highly sensitive, permitting the analysis of cAMP synthesis in only a few milligrams of tissue, lyophilization of deproteinized sample extracts after incubation with cAMP precursors could be used to increase assay sensitivity by concentrating samples prior to the HPLC purification of cAMP.

The investigations with isoproterenol and DA in the presence and absence of receptor antagonists illustrate the usefulness of this assay in studying receptor interaction with cAMP synthesis (Figs. 2—5). The use of tissue slices incubated with [^{3}H]adenine has the advantage that factors affecting cAMP metabolism can be examined using a relatively intact system. This method should also be directly applicable to use with tissue cultures. The use of ATP provides a highly sensitive assay for adenylate cyclase in broken cell preparations. Because adenosine is resolved from cAMP, [^{3}H]ATP instead of [^{32}P]ATP may be used for this type of assay. The use of high specific activity [^{3}H]ATP rather than [^{32}P]ATP has the advantage that tritium is a more stable isotope than ^{32}P, yet it still permits adequate sensitivity for most applications. When higher sensitivity is required, [^{32}P]ATP may be used and because adenosine, which is not radiolabelled when ^{32}P is used, would not interfere with the analysis, assay time may be considerably reduced by either increasing the HPLC mobile phase rates or increasing the concentration of methanol in the mobile phase (unpublished observations).

Modifications of the procedures described here also might be applied to the study of other aspects of cyclic nucleotide metabolism. For example, these procedures could be applied, as described, to purify cAMP from tissue samples prior to the analysis of endogenous cAMP levels by radioligand or enzymatic assays. In addition, preliminary investigations indicate that this methodology could be applied to studies of cyclic guanosine 3′,5′-monophosphate metabolism. Thus, these procedures provide a relatively rapid, sensitive, and versatile means for examining changes in cyclic nucleotide metabolism.

REFERENCES

J. Daly, Cyclic Neucleotides in the Nervous System, Plenum Press, New York, 1977.

2 G.A. Robinson, R.W. Butcher and E.W. Sutherland, Cyclic AMP, Academic Press, New York, 1971.
3 G. Brooker, in D. Glick (Editor), Methods of Biochemical Analysis, Wiley, New York, 1974, pp. 22, 95.
4 Y. Salomon, C. Londos and M. Rodhell, Anal. Biochem., 58 (1974) 541.
5 M. Chasin, I. Riukin, F. Mamrak, S.G. Saminiego and S.M. Hess, J. Biol. Chem., 240 (1971) 3037.
6 J. Forn and G. Krishna, Pharmacology, 5 (1971) 193.
7 G.C. Palmer, D.J. Jones, M.A. Medina, S.J. Palmer and W.B. Stavinoha, Neuropharmacology, 17 (1978) 491.
8 J. Schultz and J.W. Daly, J. Neurochem., 21 (1976) 1319.
9 H. Shimizu, J. W. Daly and C.R. Creveling, J. Neurochem., 16 (1969) 1609.
10 D.W. Schulz and R.B. Mailman, J. Neurochem., 42 (1984) 764.
11 H. Martinez-Valdez, R.M. Kothari, H.V. Hershey and M.V. Taylor, J. Chromatogr., 247 (1982) 307.
12 O.H. Lowry, N.J. Rosenbrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 581.
13 J. Bockaert, J.P. Tassin, A.M. Thierry, J. Glowinski and J. Premont, Brain Res., 122 (1977) 71.
14 J.W. Kebabian, G.L. Petzoldd and P. Greengard, Proc. Nat. Acad. Sci. U.S., 69 (1972) 2145.