Pergamon

Pharmacology Biochemistry and Behavior, Vol. 48, No. 3, pp. 813–816, 1994

0091-3057/94 $6.00 + .00

0091-3057(94)E0056-N

BRIEF COMMUNICATION

A Simple Procedure for Assaying cAMP

GNANAVEL MUNIRATHINAM AND BYRON C. YOBURN[1]

College of Pharmacy, St. John's University, Grand Central and Utopia Parkways, Queens, NY 11439

Received 8 September 1993

MUNIRATHINAM, G. AND B. C. YOBURN. *A simple procedure for assaying cAMP.* PHARMACOL BIOCHEM BEHAV **48**(3) 813–816, 1994.—A step-by-step protocol for assaying cAMP is presented. This method is based on the standard binding protein assay that is available commercially. However, using the present procedure, the per tube cost is dramatically reduced. In the current protocol, four different binding proteins are compared for their ability to bind cAMP. The source of all reagents is noted as well as necessary precautions for insuring reliable assays. A simple tissue preparation method is outlined for assaying cAMP in brain. The utility of the assay is illustrated by demonstrating the effect of forskolin on cAMP in mouse striatal tissue.

cAMP Assay Forskolin Mouse striatum Binding protein Protein kinase

cAMP is an important intracellular messenger whose formation can be both inhibited and stimulated following activation of a variety of ligand binding sites (7,9,12). A number of assay techniques are available for determining cAMP levels and for evaluating the activity of the enzyme adenylyl cyclase that converts ATP to cAMP [(2–4,10,13); see for review: (8)]. In addition, there are commercially available kits for assaying cAMP levels; however, in many cases, the cost of these kits makes their use prohibitively expensive. Therefore, in this report we present a simple, inexpensive assay for cAMP that is based on the the binding of cAMP to a protein kinase. We have demonstrated the utility of the assay by examining the effect of the direct adenylyl cyclase stimulator forskolin (6,11) on cAMP in mouse striatum. The advantage of the assay described in this report is that it is specific, simple, and inexpensive and all components are readily available.

METHOD

The assay procedure is similar to that of commercially available kits (e.g., Amersham) that depend upon the binding of cAMP to a cAMP dependent protein kinase or binding protein. The assay is based on the displacement of labeled cAMP from the binding protein by unlabeled cAMP. For construction of a standard curve, known amounts of cAMP are added to successive tubes and the amount of trace bound to the kinase is determined. Unknowns are estimated by determining the amount of trace cAMP bound to the kinase and then estimating the amount of cAMP by referring to the standard curve.

Materials

Tritiated cAMP (specific activity = 26 ci/mmole) (product #TRK304) was purchased from Amersham (Arlington Heights, IL). All other reagents were obtained from Sigma Chemical (St. Louis, MO). (Product numbers are indicated in parentheses.) cAMP dependent protein kinases were used as the binding proteins. The four kinases were derived from porcine heart (P8164), rabbit muscle (P4890; P3891 referred to in this report as rabbit muscle I and II, respectively), and bovine heart (P5511). Other compounds were: acid-washed charcoal (C4386), cAMP (A6885), ATP (A3284), cGMP (G6129), 5′AMP (A6785), GTP (G5884), EDTA (ED2SS), EGTA (E4378), BSA (A4378), theophylline (T1633), $MgSO_4$ (M1880), and forskolin (F6886). Buffers contained 50 mM Tris (pH 7.4) with 4 mM EDTA, or 50 mM TRIS with 1 mM EGTA. Binding proteins were diluted in Tris EDTA buffer to yield 4 μg or 8 μg per 100 μl with 0.1% BSA. A 3.5% charcoal slurry in Tris EDTA with 2% BSA was constantly stirred on ice. [^{3}H]cAMP, cAMP, cGMP, GTP, and 5′AMP were dissolved in Tris/EDTA. For forskolin stimulation, all reagents (theophylline, GTP, ATP, $MgSO_4$) were dissolved in TRIS/EGTA. Forskolin was initially dissolved in DMSO (D8779) (10 mg/ml) and diluted to the appropriate concentration with TRIS/EGTA buffer (5).

[1] To whom requests for reprints should be addressed.

TABLE 1

cAMP ASSAY PROTOCOL

Tube	Buffer (TRIS/EDTA)	Standard or Unknown	[^{3}H]cAMP	Binding Protein	Charcoal
Blank	150 μl	0 μl	50 μl	0 μl	100 μl
Standard	0 μl	50 μl	50 μl	100 μl	100 μl
Unknown	0 μl	50 μl	50 μl	100 μl	100 μl

The order of addition is from left to right. Tubes were assayed in duplicate in all cases. Tubes were vortexed after adding binding protein, and then stored at 2°C for 2 h. Charcoal was then added, tubes vortexed, and then centrifuged at 12,000 rpm for 7 min in a refrigerated centrifuge. A 200 μl aliquot of the supernatant was removed and counted by liquid scintillation spectrophotometry.

Procedure

The order and volume of additions to each tube is as shown in Table 1. All tubes (1.5 ml microfuge tubes) were kept on ice. Approximately 80,000 dpms of [^{3}H]cAMP was added to each tube (at above specific activity, bring 2 μl trace to 4 ml with TRIS/EDTA). Following the addition of binding protein (4 or 8 μg), tubes were vortexed (5 s) and then stored for 2 h at 2°C. Charcoal was then added to each tube, tubes were vortexed and then centrifuged (Fisher Marathon Centrifuge 21K/BR) at 12,000rpm at 5°C for 7 min. Tubes were removed from the centrifuge, carefully placed on ice such that the charcoal pellet was not disturbed, and 200 μl of supernatant was rapidly removed for counting using liquid scintillation spectrophotometry. For standard curves, 0–16 pmol of cAMP in 50 μl Tris/EDTA was added to each tube. When unknowns were assayed, 50 μl samples were employed. For determination of specificity of the assay, cGMP (10–100 nmol), GTP (50–10,000 nmol), and 5'AMP (10–100 nmol) were used. It is important to allow any ethanol in the trace to evaporate before diluting to appropriate concentration because the binding proteins appear to be sensitive to ethanol. Furthermore, binding proteins should be prepared in advance in aliquots and frozen.

TABLE 2

IC_{50}s FOR cAMP AND cGMP FOR FOUR DIFFERENT BINDING PROTEINS

Kinase	cAMP (*pmol*)	cGMP (*nmol*)
4 μg		
Porcine heart	2.92 (2.32–3.67)	0.81 (0.65–1.01)
Rabbit muscle I	2.67 (2.12–3.36)	0.25 (0.08–0.74)
Bovine heart	4.36 (3.79–5.02)	0.76 (0.69–0.83)
Rabbit muscle II	2.57 (1.86–3.55)	0.38 (0.24–0.60)
8 μg		
Porcine heart	3.43 (2.71–4.34)	
Rabbit muscle I	2.25 (1.55–3.27)	
Bovine heart	4.56 (3.33–6.23)	
Rabbit muscle II	1.68 (1.09–2.58)	

IC_{50} (95% confidence limits) values for cAMP (pmol) and cGMP (nmol) from nonlinear regression fitting of assay data for each of the four binding proteins at 4 μg (top) or 8 μg (bottom) per tube. Results are representative data from single experiments.

Data Analysis

Standard curve calculations were determined by subtracting the blank values from each standard (or unkown) tube. The number of CPMs for 0 pmol cAMP is defined as C_0. The number of CPMs for each concentration of cAMP is defined as C_x. The ratio of C_0/C_x is then plotted against pmol of cAMP (see Fig. 2). The data are fit by linear regression and unknowns can be calculated. To assess specificity of the assay, IC_{50}s for cAMP, cGMP, 5'AMP, and GTP were determined by nonlinear regression (GraphPAD ver 4.0, Graphpad Software, San Diego, CA).

Forskolin Stimulation of cAMP

Tissue (one mouse striata per tube) was rapidly removed and frozen on dry ice. Each striatum was then homgenized in ice cold 0.5 ml Tris/EGTA buffer using a motor-driven Teflon pestle and 1.5 ml microfuge tubes. Following homogenization, the volume in the tube was brought to 1 ml with Tris/EGTA buffer. A 25 μl aliquout was added to tubes containing 10 μM

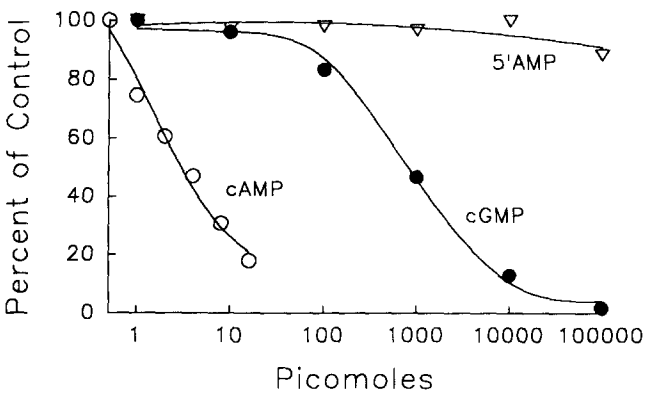

FIG. 1. Sensitivity of porcine heart binding protein (4 μg) to cAMP, cGMP, and 5'AMP.

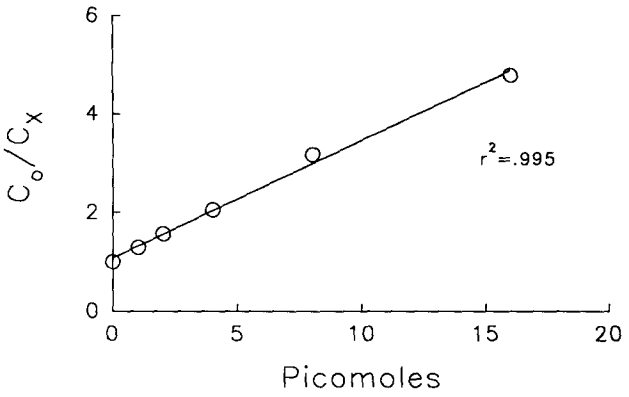

FIG. 2. Calibration curve for cAMP using porcine heart as the binding protein (4 μg). See the Method section for details.

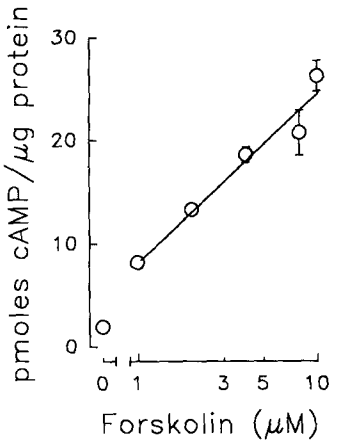

FIG. 3. The effect of forskolin on cAMP in mouse striatum determined using porcine heart as the binding protein (4 μg). Each point represents the mean (± SEM) from five to six straita.

GTP, 1 mM ATP, 3 mM $MgSO_4$, 10 mM theophylline and brought to a final volume of 1 ml with TRIS/EGTA. Tubes were incubated for 10 min (25°C) in the presence and absence of 1–10 μM forskolin. The reaction was then terminated by boiling the tubes for 5 min. Tubes were centrifuged (12,000 rpm) for 5 min at 5°C and a 50 μl aliquot of the supernatant was taken for assay for cAMP. Where appropriate, the 50 μl aliquot was diluted (e.g., 1 : 3) to insure that cAMP content would be within the range of the standard curve. Protein was determined using a microassay technique (1) using reagent purchased from BIO-RAD (Richmond, CA).

RESULTS

All four binding proteins were approximately equally sensitive to cAMP. The IC_{50}s were all less than 5 pmol, regardless of whether 4 or 8 μg of binding protein was included in the assay (Table 2). All binding proteins were significantly less sensitive to cGMP, with sensitivity ranging from ≈90- to 280-fold less compared to cAMP. Representative curves for one protein (porcine heart) are presented in Fig. 1. Neither GTP (not shown) nor 5′AMP (Fig. 1) bound significantly to any of the proteins.

To calculate unknowns, a standard curve was constructed for each protein. A representative curve for a single protein (porcine heart) is shown in Fig. 2. Unknowns can be easily calculated using the fitted linear regression.

The utility of the assay is shown in Fig. 3 in which the effects of forskolin on cAMP in mouse striatum are demonstrated. Forskolin produced a dose-dependent increase in cAMP with 10 μM forskolin producing more than a 10-fold increase over basal levels.

DISCUSSION

The present paper presents a step-by-step protocol for assaying cAMP based on the binding protein assay (4,13). The advantage of our protocol is that all reagents, including the binding proteins, were obtained commercially, thus removing the requirement for producing the binding protein. Furthermore, the cost per tube (≈$0.05/tube) is reduced substantially compared to commercially available kits. In terms of sensitivity for cAMP, the choice of binding protein, or concentration (4 or 8 μg) is not critical if one chooses among the four used here because the IC_{50}s are all less than 5 pmol. Binding of 5′AMP and GTP was negligible at concentrations up to 100 nmol (5′AMP) and 10 μmol (GTP) for all four binding proteins. The cAMP-dependent protein kinase from porcine heart had the greatest selectivity (≈280-fold) for cAMP compared to cGMP, and we have opted to use this as the binding protein (4 μg concentration) for subsequent assays. The sensitivity of the present assay compares favorably with commercially available kits.

The assay was responsive to direct stimulation of adenylyl cyclase by forskolin in mouse striatal homogenate preparations. A dose-dependent increase in cAMP was demonstrated with maximal stimulation at 10 μM being more then 10-fold. In further studies, we have observed inhibition of forskolin-stimulated cAMP increases by opioids (data not shown).

In summary, the present report details procedures for assaying cAMP based upon the well-established binding protein assay. The cost of all materials is substantially reduced compared to commercially available kits. All materials and sources are indicated so as to readily allow the assay to be established. In addition, a simple tissue preparation method is presented and the utility of the assay is demonstrated.

ACKNOWLEDGEMENTS

Our thanks to Drs. Steve Childers, Harvey Wiener, and Clint Kilts for helpful advice. Supported in part by the National Institute on Drug Abuse (DA 04185). Dr. M. T. Turnock provided helpful advice.

REFERENCES

1. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254; 1976.
2. Brown, B. L.; Albano, J. D. M.; Ekins, R. P.; Sgherzi, A. M. A simple and sensitive saturation assay method for the measurement of adenosine 3′ : 5′-cyclic monophosphate. Biochem. J. 121:561–562; 1971.
3. Childers, S. R. A high-performance liquid chromotography assay of brain adenylate cyclase using [^{3}H]ATP as substrate. Neurochem Res. 11:161–171; 1986.

4. Gilman, A. G.; Murad, F. Assay of cyclic nucleotides by receptor protein binding displacement. In: Hardmann, J. G.; O'Malley, B. W., eds. Methods in enzymology, vol. 38. New York: Academic Press; 1974:49–61.
5. Huang, R.-d.; Smith, M. F.; Zahler, W. L. Inhibition of forskolin-activated adenylate cyclase by ethanol and other solvents. J. Cyc. Nucleot. Res. 8:385–394; 1982.
6. Laurenza, A.; Sutkowski, E. M.; Seamon, K. B. Forskolin: A specific stimulator of adenylyl cyclase or a diterpene with multiple sites of action. Trends Pharmacol. Sci. 10:442–447; 1989.
7. Limbard, L. E. Receptors linked to inhibition adenylate cyclase: Additional signalling mechanisms. FASEB J. 2:2686–2695; 1988.
8. Murray, K. J. Methods in cyclic nucleotide research. In: Boulton, A.; Baker, G.; Taylor, C., eds. Neuromethods, vol. 20: Intracellular messengers. Totowa, NJ: Humana Press; 1992:313–368.
9. Northrup, J. K. Regulation of cyclic nucleotides in the nervous system. In: Siegel, G.; Agranoff, B.; Albers, R. W.; Molinoff, P., eds. Basic neurochemistry: Molecular, cellular and medical aspects, 4th ed. New York: Raven Press; 1989:349–363.
10. Salomon, Y.; Londos, C.; Rodbell, M. A highly sensitive adenylate cyclase assay. Anal. Biochem. 58:541–548; 1974.
11. Seamon, K. B.; Daly, J. W. Forskolin, cyclic AMP and cellular physiology. Trends Pharmacol. Sci. 4:120–123; 1983.
12. Taylor, P.; Insel, P. A. Molecular basis of drug action. In: Pratt, W. B.; Taylor, P., eds. Principles of drug action, 3rd ed. New York: Churchill Livingstone; 1990:103–200.
13. Tovey, K. C.; Oldham, K. G.; Whelan, J. A. M. A simple direct assay for cyclic AMP in plasma and other biological samples using an improved competitive protein binding technique. Clin. Chim. Acta 56:221–234; 1974.