Utilization of Spectral Absorption for Measurement of Adenylate Cyclase Activity

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The purpose of this study was to improve our previously described enzymatic fluorometric assay of the adenylate cyclase activity. Using physicochemical characteristics of NADPH, of which a 0.1 mmol/L solution would have an optical density of 0.627, we measured the adenylate cyclase activity by the spectral absorption of NADPH. The assay consists of two parts: pharmacological modulation of adenylate cyclase and measurement of newly synthesized cyclic AMP. The latter part involves four steps: enzymatic destruction of noncyclic adenine nucleotides and phosphorylated metabolites, conversion of cyclic AMP to ATP, amplification of ATP by enzymatic cycling, and measurement of NADPH with spectral absorption, which is

generated in proportion to initial cyclic AMP levels. This new assay was tested in membrane preparations made from rat hearts in comparison with the previously described fluorometric assay. We obtained identical results by spectrophotometry and fluorometry with high reproducibility. Because the fluorometric assay possesses a high sensitivity while the spectrophotometric method is advantageous because of its wide analytical range of cyclic AMP measurement, combination of fluorometric and spectrophotometric methods may offer a convenient way to measure the adenylate cyclase activities in various samples. *J. Clin. Lab. Anal.* 14:115–119, 2000. © 2000 Wiley-Liss, Inc.

Key words: cyclic AMP; fluorometry; spectrophotometry; NADPH; nonradioactive

INTRODUCTION

Because the classical assay of adenylate cyclase activities including radioimmunoassay (1) and Salomon method (2) are not sensitive enough to examine a small amount of tissue, in previous studies (3,4) we developed a highly sensitive, nonradioactive, enzymatic fluorometric assay technique. This enzymatic assay has a number of potential advantages: the risks and costs associated with radioactivity are eliminated; expense on a cost/assay basis is approximately 90% less than that associated with a commercially available enzyme-immunoassay or radioimmunoassay kit; no overtime incubation or counting of radioactivity is necessary, so the time required for the assay is significantly shorter compared with that of the Salomon assay; and the standard curve is linear over a wide range of cyclic AMP concentration (3,4). The purpose of this study was to explore new applications of this enzymatic assay. In this paper, we describe the utilization of spectral absorbance for the measurement of adenylate cyclase activity based on the physicochemical characteristics of NADPH, of which a 0.1 mmol/L solution would have an optical density of 0.627 (5). This new assay was tested in membrane preparations made from rat hearts in comparison with the previously described enzymatic fluorometric assay method.

MATERIALS AND METHODS

All experimentation was performed in accordance with the rules and regulations of the Committee for Research at the Yamanashi Medical University.

Production of the Membrane Preparation

The right atrium and left ventricle were obtained from male Sprague-Dawley rats weighting 200–300 g ($n = 5$). The animals were anesthetized with an intraperitoneal injection of 60 mg/kg of pentobarbital sodium. Hearts were excised and placed in ice-cold SET buffer (0.25 mol/L sucrose; 0.1 mmol/L EDTA; 5.0 mmol/L Tris-HCl, pH 7.4). These samples were homogenized with 500 μ L of ice-cold SET buffer using a pellet mixer. The mixture was centrifuged at 10,000g for 5 min at 4°C. The pellet was resuspended in the 500 μ L of SET

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buffer and the mixture was centrifuged under the same setting. After this procedure was repeated twice, the pellet was finally suspended in 100 μ L of SET buffer. Protein analysis was performed using a commercially available protein assay reagent (Pierce, Rockford, IL). The membrane suspension was diluted with SET buffer to a concentration of 2–3 mg protein/mL, and it was stored at -80°C until its adenylate cyclase activity was measured.

Measurement of Adenylate Cyclase Activity

The assay consists of two parts—namely, the production of cyclic AMP by the adenylate cyclase in the membrane preparation (part A), and the measurement of NADPH which is generated in proportion to newly formed cyclic AMP (part B) using a fully automatic microplate spectrophotometer (Molecular Devices, SPECTRA MAX 340, Sunnyvale, CA) in addition to previously described fluorescence spectrophotometer (Hitachi, F3000, Tokyo, Japan)(3,4). The results of the two different methods were compared.

Part A

This part is essentially the same as those described before (4). Forskolin was used to better confirm whether the currently used cardiac membrane preparations were functioning. Briefly, 50 μ L of adenylate cyclase mix (100 mmol/L Tris-acetate, pH 7.4; 20 mmol/L KCl; 10 mmol/L MgCl_2 ; 20 mmol/L phosphoenolpyruvate; 2 mmol/L ATP; 20 μ mol/L GTP; 2 mmol/L dithiothreitol; 0.4 mg/mL bovine serum albumin; 0.1 mmol/L 3-isobutyl-1-methylxanthine (IBMX); 100 μ g/mg pyruvate kinase) was added to each microcentrifugation tube in duplicate with or without forskolin (2×10^{-5} mol/L). Next, the membrane suspension in a volume of 50 μ L was added to each tube. The reaction mixture and membrane suspension were maintained at 4°C , both before and after being mixed, to ensure the same starting time for all assay tubes. The reaction was initiated by placing the tubes in a water bath maintained at 37°C . After 30 min, the reaction was terminated by heating at 95°C for 5 min. The mixture was vortexed 3 times and centrifuged at 10,000g for 5 min. The supernatant solution was stored for the following cyclic AMP assay.

Part B

This part is different from the previously described original method (4). We have modified several enzyme concentrations, and combined steps 2 and 3 of that method to make the assay simpler and more reliable [described in part in our recent report (6)]. The new assay consists of the following four steps. A volume of 5 μ L of the supernatant was transferred to a 10- $\times$ -75-mm disposable assay tube (Iwaki Lab Ware, Tokyo, Japan) in triplicate. For the cyclic AMP standard, 5 μ L of a known amount of cyclic AMP was added to the tubes.

Step 1

A volume of 25 μ L of reaction mix A (100 mmol/L Tris-HCl, pH 8.0; 2 mmol/L MgCl_2 ; 4 units/mL apyrase; 10 units/mL adenosine deaminase; 40 units/mL alkaline phosphatase) was added to each tube. The mixture was incubated at 37°C for 5 min to destroy potential interfering substances including noncyclic adenine nucleotides and phosphorylated metabolites. Enzymes were then destroyed by heating for 3 min at 95°C .

Step 2

A volume of 25 μ L of reaction mix B (100 mmol/L Tris-HCl, pH 8.0; 2 mmol/L MgCl_2 ; 0.1 mg/mL bovine serum albumin; 150 mmol/L KCl; 2 mmol/L dithiothreitol; 40 nmol/L ATP; 8 mmol/L phosphoenolpyruvate; 12 units/mL phosphodiesterase; 4.5 units/mL myokinase; 80 units/mL pyruvate kinase) was added to each tube from step 1. The mixture was incubated for at least 6 hr at room temperature to completely convert cyclic AMP to ATP. This was usually performed by an overnight incubation.

Step 3

A volume of 25 μ L of reaction mix C (100 mmol/L Tris-HCl pH 8.0; 2 mmol/L MgCl_2 ; 0.1 mg/mL bovine serum albumin; 8 mM fructose; 60 units/mL hexokinase) was added to the tubes from step 2 on ice. The mixture was incubated for 1 hr at 37°C to amplify ATP to about 1,000 $\times$ of fructose-6-phosphate.

Step 4

A volume of 125 μ L of reaction mix D (100 mmol/L Tris-HCl, pH 8.0; 3.2 mmol/L EDTA; 0.6 mmol/L NADP^+ ; 1 units/mL phosphoglucose isomerase; 0.5 units/mL glucose-6-phosphate dehydrogenase) was added to the tubes from step 3. After the mixture was incubated for 10 min at room temperature, a volume of 600 μ L of distilled water was added to each tube.

The concentration of NADPH, formed when the glucose-6-phosphate was converted to 6-phosphogluconolactone, was measured using the fluorescence spectrophotometer as an emission fluorescence of 460 nm by the 340 nm of excitation wave. Then, each final mixture in a volume of 300 μ L was transferred to a 96-well microtest plate (Becton Dickinson, Franklin Lakes, NJ). The 340-nm absorbance of the same final mixture was measured using the full automatic spectrophotometer.

Biochemicals

Apyrase, phosphoenolpyruvate, phosphodiesterase, IBMX, sucrose, fructose, dithiothreitol, and forskolin were obtained from Sigma Chemical (St. Louis, MO). All other enzymes and substrates were obtained from Boehringer-Mannheim (Indianapolis, IN).

Statistics

The data are presented as mean $\pm$ SE. The statistical comparisons of mean values between the methods were evaluated by *t*-test for paired data. A correlation between two groups was assessed using Pearson's correlation coefficient. A *P* value less than 0.05 was considered significant.

RESULTS

Determination of Linear Range of the Experiment for Measuring NADPH With Spectral Absorption and Fluorescence

The emission fluorescence of 460 nm by the 340 nm of excitation wave was measured using a known amount of NADPH in concentration of 0–0.96 mmol/L in triplicate to assess the range of linearity. As shown in Figure 1A, the fluorescence was linear over 0–0.1 mmol/L of NADPH. Then, the spectrophotometric absorbance of the same final mixture at 340 nm was measured for the same purpose. As shown in Figure 1B, the absorbance was linear over 0–0.5 mmol/L of NADPH.

Measurement of the Same Cyclic AMP Standard With Spectral Absorption and Fluorescence for Determining the Analytical Sensitivity

A representative standard curve of cyclic AMP in concentrations of 0 to 7.6 pmol/tube with fluorescence is shown in Figure 2A, and that with spectral absorption is depicted in Figure 2B. The standard curve with fluorescence showed a slope of 2.1 and the fluorescence reading ranged from 6.0 to 21.6 arbitrary fluorescence units. On the other hand, the standard curve with spectral absorption showed a slope of 0.086 and the absorbance reading ranged from 0.224 to 0.289, indi-

cating that total amplification of 1,100 has been made. The correlation between the fluorescence reading and the spectral absorbance is summarized in Figure 2C. A linear correlation was observed between these two different methods.

Measurement of the Adenylate Cyclase Activities of Cardiac Membrane Preparations With Spectral Absorption and Fluorescence

The results of adenylate cyclase activities measured with fluorescence and spectral absorption are summarized in Figure 3. Forskolin significantly increased the adenylate cyclase activity of the atrium as well as the ventricle; however, no difference was detected between the results obtained by these two different methods. The analytical range of each method was wide enough to measure all samples without predilution. The results of multiple analysis of a single sample by either method did not differ by more than 10%, with variability usually less than 5%. According to a standard National Committee for Clinical Laboratory Standards (NCCLS) document (7), the assay precision is estimated by a replication experiment, in which the same sample material is analyzed at least 20 times by each assay to obtain the standard deviation (SD). In the present study, the basal and forskolin-stimulated adenylate cyclase activities were measured 20 times in one ventricular tissue preparation. The basal and stimulated activities (pmol/min/mg protein, mean $\pm$ SD) obtained by the spectral absorption method were 51.1 ± 0.8 and 92.7 ± 1.5 , respectively. These SDs can be considered acceptable when compared with the SDs of 1.5 and 3.7 obtained by the fluorescence method.

DISCUSSION

Because the nonradioactive, enzymatic fluorometric assay has a number of potential advantages as already shown in

Fig. 1. Optimal range for measuring NADPH with fluorescence reading (A) and spectral absorption (B). Data are expressed as mean $\pm$ SE (*n* = 3).

Fluorescence, an emission fluorescence of 460 nm by the 340 nm of excitation wave.

Fig. 2. Measurement of the same cyclic AMP standard with fluorescence reading (A) and spectral absorption (B). Correlation between spectral absorption and fluorescence reading of the same cyclic AMP standard (C). Data are expressed as mean $\pm$ SE ($n = 3$). Fluorescence, an emission fluorescence of 460 nm by the 340 nm of excitation wave.

previous reports (3,4), the present study explored the possibility for the enzymatic fluorometric assay of the adenylate cyclase activity to be used easily and widely. We found that cyclic AMP can be measured by the spectral absorption of NADPH using the same procedure as that of the fluorometric assay method, and we found identical adenylate cyclase activity from the same cardiac preparations by these two methods. This is the first report describing the measurement of adenylate cyclase activity with spectral absorbance.

The dose-response curve of NADPH was linear only over the concentrations of 0–0.1 mmol/L for fluorometric assay, but it was linear over the concentrations of 0–0.5 mmol/L for spectral absorption. These results indicate that cyclic AMP can be measured in the range of 0–25 pmol/assay by fluorometric assay and in the range of 0–200 pmol/assay by spectral absorption. Thus, the measurement with spectral

Fig. 3. Measurement of the adenylate cyclase activities of cardiac membrane preparations with spectrophotometry and fluorometry. Adenylate cyclase activities were measured with and without forskolin (10^{-5} mol/L). Data are expressed as mean $\pm$ SE ($n = 5$).

absorption may possess potential utilities for a wider analytical range of cyclic AMP measurement. Moreover, the spectrophotometric assay may possess several advantages: the spectrophotometer is more readily found in many basic and clinical laboratories than is the fluorescence spectrophotometer, and use of commercially available microtest plates together with the automatic spectrophotometer will shorten the time required for the measurement of the absorbance of the final mixtures.

Another important finding is the difference of the sensitivity of each method. The arbitrary unit of 0.1 reflects 50 fmol/assay of cyclic AMP in the fluorometric assay, while 0.01 reflects 1.2 pmol/assay of cyclic AMP in the spectrophotometric assay. This indicates that the minimum detectable concentration of the fluorometric assay is 10 nmol/L, and that of the spectrophotometric assay was 240 nmol/L. These results are in accordance with those from previous studies (5); the upper limit for fluorometry is about equal to the lower limit for accurate spectrophotometry with conventional equipment. Moreover, to better assess the analytical sensitivity of these assays, we compared their concentrations corresponding to two standard deviations above the mean signal from replicate measurement of the zero calibrator. The concentrations were 0.429 pmol/assay (86 nmol/L) for the fluorometric assay and 0.926 pmol/assay (185 nmol/L) for the spectral absorption method. Thus, the fluorometric method can be characterized as having a higher level of analytical sensitivity and a lower detection limit than does the spectral absorption method.

In summary, we obtained essentially the same assay results (with similar levels of precision) using either spectrophotometry or fluorometry. The fluorometric assay offers a higher sensitivity, while the spectrophotometric method of-

fers a wider analytical range of cyclic AMP measurement. A combination of fluorometric and spectrophotometric methods may become a convenient way to measure the adenylate cyclase activities in various types of samples.

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