Fluorescent Assay of Cyclic Nucleotide Phosphodiesterase Activity in a Neutral Aqueous Solution

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Received October 14, 2012, Accepted October 27, 2012

Key Words: Cyclic nucleotide phosphodiesterase, Fluorescence, Cyclic flavin mononucleotide, Flavin mononucleotide

Cyclic nucleotide phosphodiesterase (PDE) hydrolyzes the intracellular second messengers, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), to adenosine monophosphate (AMP) and guanosine monophosphate (GMP).¹ Eleven families of PDE have been reported in mammals² and affect cellular activity in the brain, heart, lungs, and muscles.³ Selective inhibitors of the different PDE isoforms have great potential to be used as novel therapeutic agents in areas such as hypertension, congestive heart failure, thrombosis, glaucoma, and asthma.⁴ Among them, the PDE3 inhibitor is used in the treatment of acute heart failure and the PDE5 inhibitor is used to treat erectile dysfunction.

As the inhibition of PDE activity is clinically important, significant effort has been invested in real-time analysis of the PDE enzyme reaction.⁵ The radiometric method⁶ and HPLC have been used for the assay of PDE. However, the radiometric method is laborious in practice because it requires an isotopically labeled substrate and selectivity of adsorption on the resin, and HPLC analysis does not enable real-time monitoring. Therefore, a fluorescence assay is necessary for the real-time monitoring of enzymatic reactions. Until now, the reported fluorometric detection methods of PDE activity⁷ have been used only for the hydrolysis of cAMP⁸ and cGMP.⁹ Herein we report a chemosensor-based fluorescent real-time assay of PDEs that hydrolyze riboflavin 4',5'-cyclic phosphate (cyclic FMN = cFMN) to flavin mononucleotide (FMN) in a neutral aqueous solution.

The identification of new biological pathways is significant and challenging.¹⁰ Several fluorescent chemosensors¹¹ have been developed to probe or uncover new biological reactions, which would not have been possible with HPLC or other bioanalytical methods. Recently, several research groups have discovered new biological pathways related to cFMN.¹² cFMN is known as a product of an enzyme reaction, FAD (flavin adenine dinucleotide) cFMN + AMP catalyzed by FMN cyclase.¹³ Cameselle and coworkers reported fluorometric HPLC detection of endogenous cFMN in rat liver.¹⁴ However, no assay has been developed to detect FMN produced by the enzymatic reaction of cFMN catalyzed by PDE.

In this paper, we report the first real-time fluorescent monitoring of the hydrolysis reaction of cFMN catalyzed by PDE using the chemosensor PTZ-DPA (phenothiazine-Zn(II)-dipicolylamine complex), which subsequently enables the label-free fluorescent real-time assay of PDE.¹⁵ PTZ-DPA consists of phenothiazine and bis(Zn²⁺-dipicolyl amine), which binds strongly to the phosphate of FMN (Scheme 1). The isoalloxazine ring of FMN emits green fluorescence (quantum yield: 0.26, excitation wavelength: 445 nm, emission wavelength: 525 nm) in water.¹⁶ The fluorescence emission inherent in FMN due to the isoalloxazine moiety is effectively quenched upon binding of the phosphate group of FMN to PTZ-DPA because the phenothiazine group of PTZ-DPA acts as an electron donor and quenches the emission from the isoalloxazine moiety in the complex by photoinduced electron transfer (PET).¹⁷ We were able to conduct fluorescence monitoring of PDE type IV and type VI activity in a neutral aqueous solution at 25 ºC by following the decrease in the fluorescence intensity of FMN. However, the substrate (cFMN) emits its original fluorescence, as the cyclic phosphate moiety of cFMN is extremely weakly bound to PTZ-DPA.

PTZ-DPA was synthesized according to the reported method.¹⁵ The PDE activity was investigated by fluorescent time-course measurement assay. To the pH 7.2 HEPES (10 mM) buffer solution containing MgCl₂ (1 mM), cFMN (10 µM), and PTZ-DPA (50 µM), PDE type IV and type VI were added at 25 ºC in different concentrations (0.25 mg/
of PDE activity by a chemosensor. The method introduced to FMN by PDE.

Two peaks is 18 Da, which supports the hydrolysis of cFMN due to the PDE activity. The difference in decreased and that (Figure S1(c) shows the PDE inhibition activity of PDE type IV and PDE type VI was performed in a mixture of cFMN (100 μM), PTZ-DPA (50 μM), and IBMX (100 μM) in a pH 7.2 buffer solution (10 mM HEPES, 1 mM MgCl2). The stock solution of PDE was added to afford a final concentration of 1 mg/mL. PDE type IV also showed a more dramatic change than PDE type VI (Figure S1(a) and (b)). Figure S1(c) shows the PDE inhibition activity of IBMX at various concentrations (0, 10, 50, 100 μM). The decrease in the PDE enzymatic activity can be monitored by the change in fluorescence.

We were able to prove that PDE hydrolyzes cFMN to FMN by CE/ESI-MS (capillary electrophoresis/electro-spray ionization mass spectrometry). CE/ESI-MS is a rapid, high-resolution and high-efficiency method for the simultaneous detection of compounds, and we used it to follow the appearance of the peak of FMN as cFMN reacted with PDE. cFMN (100 μM) and PDE type IV (10 mg/mL solution containing 1 mM of MgCl2) were incubated at 37 °C for 1 h. Figure S2 shows that the intensity (m/z 437) of cFMN decreased and that (m/z 455) of FMN increased dramatically due to the PDE activity. The difference in m/z between the two peaks is 18 Da, which supports the hydrolysis of cFMN to FMN by PDE.

In conclusion, we presented real-time fluorescent detection of PDE activity by a chemosensor. The method introduced in this paper gives a convenient and real-time fluorometric assay of the hydrolysis of cFMN to FMN by PDEs. Moreover,

the chemosensor can be successfully used for screening of a PDE inhibitor. Label-free fluorometric assaying of PDE and screening of PDE inhibitors has great potential in the identification of new biological pathways.

Acknowledgments. This work was supported by the NRF grant funded by the MEST (Grant No. 2012-000159).

References