Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry Letters 13 (2003) 1079–1082

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Visual Detection of AMP and Real-Time Monitoring of Cyclic Nucleotide Phosphodiesterase (PDE) Activity in Neutral Aqueous Solution. Chemosensor-Coupled Assay of PDE and PDE Inhibitors

Min Su Han and Dong H. Kim*

Center for Integrated Molecular Systems and Department of Chemistry, Division of Molecular and Life Sciences, Pohang University of Science and Technology, San 31 Hyojadong, Pohang 790-784, South Korea

Received 24 October 2002; accepted 8 January 2003

Abstract—A phosphate chemosensor ensemble, $[Zn(H\text{-}bpmp)(pyrocatechol\ violet)]^+$ is useful for a real-time assay of phosphodiesterases (PDEs) in a neutral aqueous solution. In addition, a simple and convenient screen and assay procedures for inhibitors of PDEs have been demonstrated using IBMX, a nonselective PDE inhibitor as an example.

The family of cyclic nucleotide phosphodiesterases (PDEs) represents a number of hydrolases responsible for the degradation of cyclic adenosine 3′,5′-monophosphate (cAMP) and cyclic guanosine 3′,5′-monophosphate (cGMP) to form 5′-monophosphate of the corresponding nucleotides, that is, AMP and GMP.[1] cAMP and cGMP relate the extracellular signals to cellular function in the signal transduction process.[2] To date, eleven families of PDEs have been characterized based on their amino acid sequences, endogenous regulators and substrate specificities.[2] Inhibition of PDEs exerts a broad spectrum of effects on the activities of immune and inflammatory cells, and these enzymes have served as targets for drug design.[3] Several PDE4 inhibitors are currently under clinical evaluation as antiasthmatic agents.[4] Sildenafil that is being used for the treatment of male erectile dysfunction is an inhibitor of PDE5.[5]

Assay and kinetic evaluations of PDEs are generally performed by the radiometric method that uses tritium-labeled cAMP as substrate.[6] Fluorescent derivatives of cyclic nucleotide are also used as substrates. In these cases, the fluorescence changes that accompany the hydrolysis of the cyclic phosphate are measured.[7] Alternatively, the PDE reaction is coupled to 5′-nucleotidase that converts the AMP generated by PDEs to adenosine and phosphate, and the formation of the latter phosphate is followed.[8] Newton et al. have reported the kinetic analysis of PDEs by the positive-ion fast-atom bombardment (FAB) mass spectrometry.[9] These protocols are, however, laborious in practice and require isotopically labeled substrate or substrate conjugated to a fluorescent moiety, and expensive instrumentation. We wish to report herein an operationally simple new PDE assay protocol enables monitoring of PDE activity in real-time. In this assay an AMP chemosensor is linked to PDE reaction.

The present protocol makes use of the phosphate probe prepared by dissolving 2,6-bis[bis(2-pyridylmethyl)aminomethyl]-4-methylphenol (H-bpmp), zinc perchlorate, and pyrocatechol violet in water of neutral pH in an 1:2:1 molar ratio.[10] In the probe, $[Zn_2(H\text{-}bpmp)]^{3+}$ functions as a receptor for a phosphate ion and pyrocatechol violet is the chromogenic signaling unit. It was thought that the phosphate moiety in the AMP that is generated by PDE-catalyzed hydrolysis of cAMP would displace the receptor-bound pyrocatechol violet by successfully competing for the receptor with the signaling unit, and the displacement is communicated spectrophotometrically.[11] Figure 1 depicts schematically the rationale involved in the present PDE assay protocol.

*Corresponding author. Tel.: +82-54-279-3101; fax: +82-54-279-5877; e-mail: dhkim@postech.ac.kr

0960-894X/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0960-894X(03)00055-6

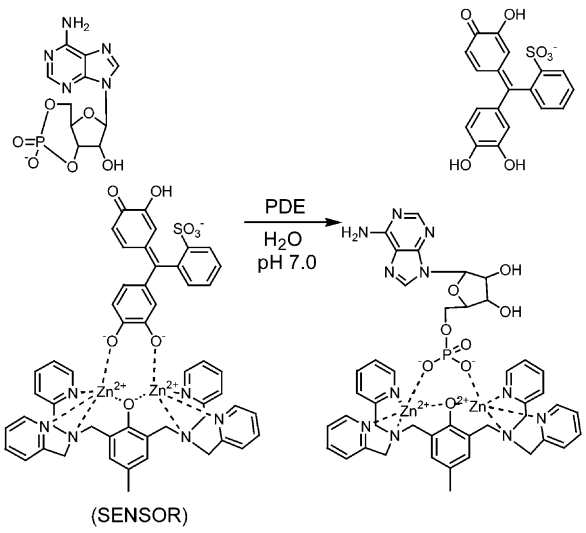

Figure 1. Schematic representation illustrating the PDE assay using an AMP chemosensor.

We first evaluated the phosphate probe as a probe for AMP. Additions of AMP to the pH 7.0 (HEPES 10 mM, $MgCl_2$ 1 mM) buffer solution containing $[Zn_2(H\text{-}bpmp)(pyrocatechol\ violet)]^+$ resulted in a change in the UV–vis absorption spectra: while the absorbance at $\lambda_{max} = 620$ nm that is due to the receptor-bound pyrocatechol violet decreases, the absorbance at 444 nm increases.[12] The latter absorbance is ascribed to the free pyrocatechol violet generated by the displacement from the receptor by AMP. Figure 2a shows the UV–vis spectral change when the solution of AMP was titrated into the aqueous solution of the probe, and Figure 2b is replot of the UV–vis absorbance at 620 nm against the concentration of AMP, showing that the absorbance decreases linearly as the concentration of AMP increases. No such changes were observed upon the additions of cAMP to the sensor solution.

The thermodynamic parameters and association constant for the binding of AMP to the receptor were determined by the isothermal titration calorimetry (ITC).[13] Figure 3 shows the ITC plot for the titration of $[Zn_2(H\text{-}bpmp)]^{3+}$ with AMP at 30 °C in an aqueous medium of pH 7.0, from which the molar enthalpy (ΔH^O) for the binding interactions was obtained to be -4.80 kcal mol^{-1}, and the entropy change (ΔS^O) was calculated to be $+8.9$ eu using the Gibbs–Helmholtz equation. The thermodynamic parameters suggest that the binding of AMP to the receptor is essentially enthalpy driven. The K_{ass} value of $(2.51 \pm 0.74) \times 10^5$ M^{-1} was calculated using the equation, $\Delta G^O = -RT \ln K_{ass}$, in which R and T represent the gas constant and absolute temperature, respectively, revealing that the analyte binds the receptor more tightly than the signaling unit.[14]

Use of the sensor for assaying the enzymic activity of PDE was investigated. To the pH 7.0 HEPES (10 mM) buffer solution containing $MgCl_2$ (1 mM), cAMP (0.1 mM) and the sensor (50 μM) was added various concentrations of PDEs[15] at 25 °C and the decrease in the UV–vis absorbance at 620 nm was monitored to obtain Figure 4, suggesting that the sensor is operative in real time. Replot of the initial rate against concentration of PDEs afforded a straight line (inset in Fig. 4), which indicates that the apparent reaction rate increases in proportion to enzyme concentration. That the enzyme reaction is the rate limiting process in the assay is thus demonstrated.

The sensor may be used for screening and assaying of PDE inhibitors. This has been demonstrated using 3-isobutyl-1-methylxanthine (IBMX), a potent non-selective PDE inhibitor having the IC_{50} value in the range of μM conentration.[16] To the assay mixture containing cAMP (100 μM), the probe (50 μM), and various concentrations of IBMX in a pH 7.0 buffer solution (10 mM HEPES, 1 mM $MgCl_2$) was added the stock solution of PDEs to afford the final concentration of 0.0184 U mL^{-1}. Figure 5 shows the enzymic activity of PDEs in the presence of various concentrations of IBMX, indicating that the loss of the enzymic activity caused by IBMX can be monitored by the change of UV–vis absorbance. Replot of percent inhibition against concentration of the inhibitor gave a rectangular

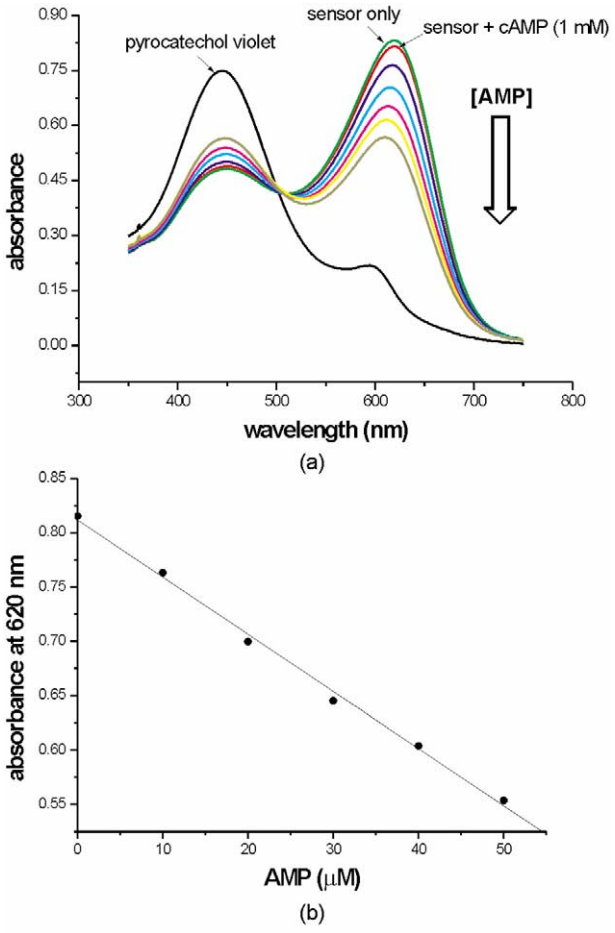

Figure 2. (a) UV spectra changes upon additions of AMP stock solution (final concentration of AMP: 0, 10, 20, 30, 40, and 50 μM) to the pH 7.0 aqueous buffer (10 mM HEPES, 1.0 mM $MgCl_2$) containing the AMP sensor (50 μM); (b) Replot of UV absorbance at 620 nm against concentration of AMP.

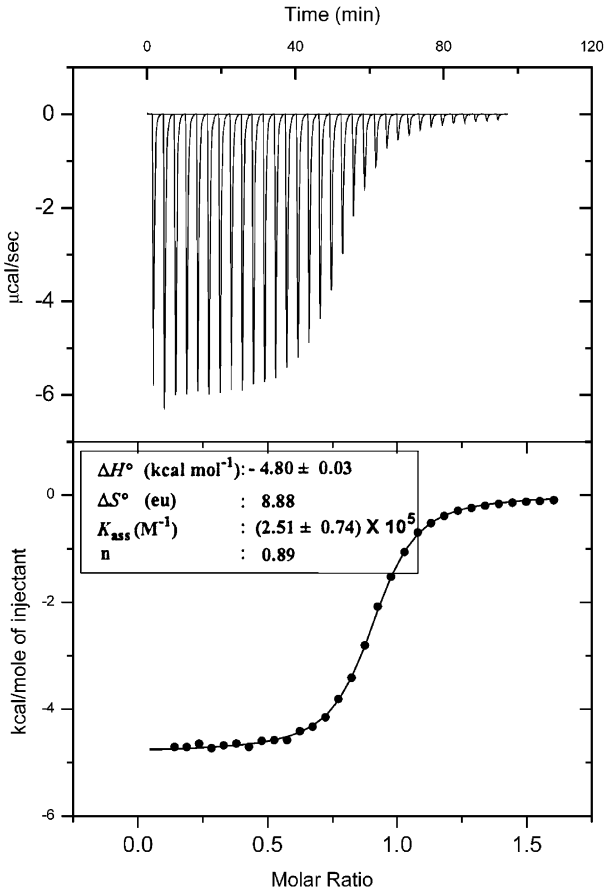

Figure 3. The ITC plot for the titration of $[Zn_2(H\text{-bpmp})]^{3+}$ (0.5 mM) with AMP (5 mM) in an aqueous buffer pH 7.0 at 30°C. An aqueous solution (1.5 mL, pH 7.0, HEPES 10 mM) of $[Zn_2(H\text{-bpmp})]^{3+}$ (0.5 mM) was added to the calorimeter cell. To this solution was injected a 7 µL portion of aqueous AMP solution (5 mM) 32 times. The mixture was continuously stirred and was kept at an operating temperature of 30 °C. The data were analyzed and fitted using the software Origin. Inset: Thermodynamic parameters (ΔH^O, ΔG^O and ΔS^O), association constant (K_{ass}), and stoichiometry (n) for the binding of AMP to $[Zn_2(H\text{-bpmp})]^{3+}$ in aqueous solution of pH 7.0 (HEPES 10 mM).

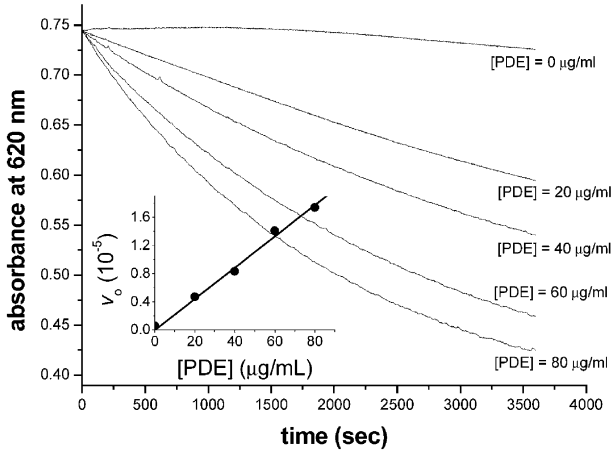

Figure 4. PDEs catalyzed hydrolysis of cAMP to AMP was monitored at pH 7.0 with the sensor by measuring the UV absorption at 620 nm. Inset: replot of the initial rate against concentration of PDEs.

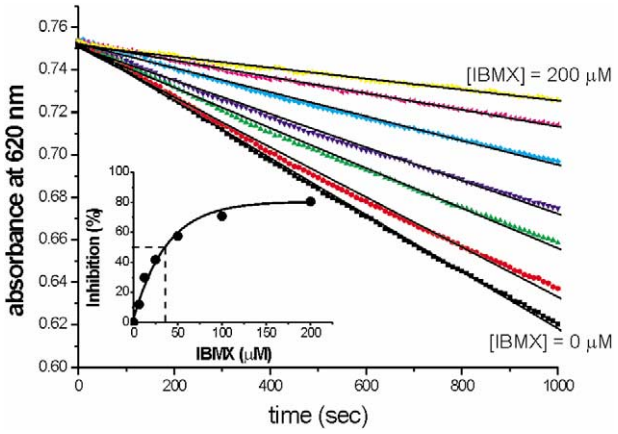

Figure 5. Inhibition of PDEs with IBMX, a nonselective PDE inhibitor: Plot of UV absorbance at 620 nm against time, showing the intensity of the absorbance decrease by additions of IBMX (final concentrations: 0, 6.25, 12.5, 25.0, 50.0, 100, and 200 µM) to the pH 7.0 buffer solution (HEPES 10 mM, $MgCl_2$ 1 mM) containing cAMP (100 µM), PDEs (0.018 U/mL), and the sensor (50 µM). Inset: Replot of percent inhibition of PDEs against the concentration of IBMX, from which the IC_{50} value for IBMX is estimated.

hyperbolic curve (inset), from which the IC_{50} value for the inhibition of PDEs with IBMX was estimated to be 36 µM.

High-throughput screening (HTS) is now widely used for identification of hit compounds from combinatory library. We have examined the applicaton of the probe for HTS of PDE inhibitors by visual inspection. The catalytic hydrolysis of the cyclic phosphate by PDE in the presence of $[Zn_2(H\text{-bpmp})(\text{pyrocatechol violet})]^+$ accompanies the color change from blue to yellow: no color change hence indicates inhibition of PDEs. Figure 6 shows the color of wells that contains PDEs, cAMP, and the phosphate sensor in the presence (blue) and absence (yellow) of IBMX. The inhibition of PDEs is communicated by blue color.[17]

In conclusion, we have demonstrated that $[Zn_2(H\text{-bpmp})(\text{pyrocatechol violet})]^+$ is useful as a chemosensor for the detection of AMP, and developed a convenient and real time spectrophotometric assay protocol for PDE that hydrolyzes cAMP to AMP by coupling the enzymic activity to the chemosensor. Furthermore, the application of the chemosensor for screening and

Figure 6. A microtiter well (right) that contains cAMP (500 µM), PDEs (0.023 µ/mL), the sensor (50 µM) and IBMX (500 µM) is colored blue but the well (left) that contains the same mixture except IBMX is yellow. The picture was taken after incubating the mixture for 1 h at room temperature.

assaying of PDE inhibitors has been demonstrated using IBMX, a nonselective PDE inhibitor. The present assay methods for PDE and PDE inhibitors employ unlabled cAMP as the substrate.

Acknowledgements

The work was supported by Korea Science and Engineering Foundation.

References and Notes

1. (a) Buther, R. W.; Sutherland, E. W. *J. Biol. Chem.* **1962**, *237*, 1244. (b) Beavo, J. In *Cylic Nucleotide Phosphodiesterases: Structures, Regulation and Drug Action*, Beavo, J., Houslay, M. D. Eds.; John Wiley: Chichester, 1990; p 3.
2. Mehats, C.; Andersen, C. B.; Filopanti, M.; Jin, S.-L.C.; Conti, M. *Trends Endocrinol. Metabol.* **2002**, *13*, 29.
3. Schudt, C., Dent, G., Rabe, K. F., Eds. *Phosphodiesterase Inhibitors*. Academic Press: New York, 1996.
4. Huang, Z.; Duckarme, Y.; Macdonald, D.; Robichaud, A. *Curr. Opin. Chem. Biol.* **2001**, *5*, 432.
5. Terrett, N. K.; Bell, A. S.; Brown, D.; Ellis, P. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 1819.
6. (a) Thompson, W. J.; Appleman, M. M. *Biochemistry* **1971**, *10*, 311. (b) Lynch, T. S.; Cheung, W. Y. *Anal. Biochem.* **1975**, *67*, 130. (c) Gulyassay, P. F.; Farrand, J. R. *J. Chromatogr.* **1976**, *129*, 107. (d) Ledonne, N. C., Jr.; Coffee, C. J. *J. Biol. Chem.* **1979**, *254*, 4317. (e) Memoz, G.; Sette, C.; Conti, M. *Mol. Pharmacol.* **1997**, *51*, 242.
7. (a) Hiratsuka, T. *J. Biol. Chem.* **1982**, *257*, 13354. (b) Johnson, J. D.; Walters, J. D.; Mills, J. S. *Anal. Biochem.* **1987**, *162*, 291.
8. (a) Leung, P. C.; Taylor, W. A.; Wang, J. H.; Tipton, C. L. *J. Biol. Chem.* **1984**, *259*, 2742. (b) Au, T. K.; Wallace, S. H.; Leung, P. C. *Int. J. Biochem. Cell Biol.* **2000**, *32*, 1173.
9. Newton, R. P.; Bayliss, M. A.; Khan, J. A.; ; Bastani, A.; Wilkins, A. C. R.; Games, D. E.; Walton, T. J.; Brenton, A. G.; Harris, F. M. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 574.
10. Han, M. S.; Kim, D. H. *Angew. Chem. Intl. Ed.* **2002**, *41*, 3809.
11. Very recently, Mizukami et al. (Mizukami, S.; Nagano, T.; Urano, A.; Kikuzi, K. *J. Am. Chem. Soc.* **2002**, *124*, 3920) have reported that a chemosensor consisting of Cd(II)-cyclen (receptor) and 7-amino-4-trifluoromethylcoumarin (signaling unit) is effective for the detection of pyrophosphate, citrate, ATP, or ADP and demonstrated that it is useful for fluorescence assay of PDE in an aqueous buffered solution of pH 7.4.
12. The phosphate sensor was shown to exert most sensitive response at pH 7.0.[10]
13. Jelesarov, I.; Bosshard, H. R. *J. Mol. Recog.* **1999**, *12*, 3.
14. The K_{ass} value for pyrocatechol violet binding to the receptor was reported to be $(5.3\pm0.2)\times10^4$ M^{-1}.[10]
15. Commercially available PDEs (Sigma PO134, approx. 0.2 unit per mg protein) isolated from bovine heart (Butcher, R. W.; Sutherland, E. W. *J. Biol. Chem.* **1962**, *237*, 1244) were used without further purification. One unit hydrolyzes 1.0 μmol of 3′,5′-cAMP per min at pH 7.5 at 30 °C.
16. (a) Beavo, J. A.; Rogers, N. L.; Crofford, O. B.; Hardman, J. G.; Sutherland, E. W.; Newman, E. V. *Mol. Pharmacol.* **1970**, *6*, 597. (b) Sekhar, K. R.; Grondin, P.; Francos, S. H.; Corbin, J. D. In *Phosphodiesterase Inhibitors*; Schudt, C., Dent, G., Rabe, K. F. Eds.; Academic: New York, 1996; p 137.
17. Lately, Huang et al. (Huang, W.; Zhang, Y.; Sportman, J. R. *J. Biomolecular Sensing* **2002**, *7*, 215) reported a PDE assay protocol that is based on IMAP technology and uses fluorecein-linked cAMP or cGMP as substrate. This method is shown to be amenable to HTS of PDE inhibitors.