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A single electrochemical biosensor for detecting the activity and inhibition of both protein kinase and alkaline phosphatase based on phosphate ions induced deposition of redox precipitates

Congcong Shen [a], Xiangzhi Li [a], Avraham Rasooly [b], Linyan Guo [a], Kaina Zhang [a], Minghui Yang [a,*]

[a] College of Chemistry and Chemical Engineering, Central South University, Changsha 410083, China
[b] National Cancer Institute, National Institutes of Health, Rockville, MD 20850, USA

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ABSTRACT

Protein kinase (PKA) and alkaline phosphatase (ALP) are clinically relevant enzymes for a number of diseases. In this work, we developed a new simple electrochemical biosensor for the detection of the activity and inhibition of both PKA and ALP. One common feature of the PKA and ALP catalyzing process is that PKA can hydrolysis adenosine-5′-triphosphate (ATP) and ALP can hydrolysis pyrophosphate, both reactions produce phosphate ions, and the amount of phosphate ion produced is proportional to enzyme activity. Our assay is based on the principle that phosphate ions react with molybdate to form redox molybdophosphate precipitates on the electrode surface, thus generating electrochemical current. The detection limit for PKA and ALP were much lower than existing assays. The biosensor has good specificity and was used to measure drug-stimulated PKA from lysates of HeLa cells. We also evaluated the use of the biosensor as a screening tool for enzyme inhibitors. To the best of our knowledge, this is the first report of a biosensor capable of detecting the activity of both PKA and ALP. This tool has the potential to simplify PKA and ALP clinical measurement, thereby improving diagnostics of relevant diseases. It also may serve as the basis for a simple screening method for new enzyme inhibitors for disease treatment.

1. Introduction

Enzymes are biological catalysts that participate in almost all metabolic processes in the cell (Luo et al., 2015; Sallmann and Limberg, 2015; Zhang and Houk, 2005; Kang et al., 2015). Enzymes serve a wide variety of functions inside living organisms. Monitoring activity of enzymes and discovering new enzyme inhibitors are of great importance in biomedical research, clinical diagnosis and treatment.

Among the various enzymes, protein kinase (PKA) and alkaline phosphatase (ALP) are two widely studied families of enzymes (Dong et al., 2015; Liu et al., 2014a; Qian et al., 2015a). PKA is responsible of transferring phosphate groups from nucleoside triphosphates (usually adenosine-5′-triphosphate, ATP) to specific amino acids, thus catalyzing the phosphorylation of proteins (Lee et al., 2015; Li et al., 2013b; Shin et al., 2014; Wang et al., 2013). ALP catalyzes the dephosphorylation of proteins (Barrozo et al., 2015; Yang et al., 2016; Yu et al., 2015). Phosphorylation and dephosphorylation processes lead to the functional change of the target proteins, which play vital roles in regulating intracellular processes involved in cell cycle, growth, apoptosis, and signal transduction (Chooi et al., 2014; Zhang et al., 2013).

Aberrant PKA and ALP levels are associated with several diseases (Sims et al., 2013). PKA is the best understood member of the serine-threonine protein kinase super family, and is involved in the regulation of a variety of cellular processes. It has been implicated in the initiation and progression of many tumors and, therefore, has been proposed as a novel molecular target for cancer therapy (Naviglio et al., 2009b; Sapio et al., 2014). A number of specific protein kinase inhibitors are already in use as cancer therapeutic agents (Davies et al., 2000). ALP is clinically important for liver, bone and kidney physiology (Weiss et al., 1988). ALP testing is used to help detect liver diseases (Stepien et al., 2016), cancer (Lopez et al., 1996) and bone disorders (Hessle et al., 2002). Because of the substantial clinical relevance of PKA and ALP testing, several analytical assays were developed to measure their activities.

The assays of PKA and ALP activities are typically performed by incubation of the enzymes with specific substrate and then the interaction of the substrates with various signal probes (Kang

* Corresponding author.
E-mail address: yangminghui@csu.edu.cn (M. Yang).

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et al., 2014; Wang et al., 2015a). For example, Nie and coworkers reported a method for the detection of PKA activity by initially phosphorylation of peptide substrate using PKA, then the following interaction of the phosphopeptides with semisynthetic green fluorescent protein resulted in the fluorescence enhance of the proteins (Yin et al., 2015a). Upon the assay of enzyme activity, another important job is the screening of enzyme activity inhibitors as these inhibitors can be used as potential drugs for the treatment of relevant diseases (Li et al., 2013a; Wang et al., 2015c; Yin et al., 2015b). Different methods have been reported for PKA and ALP detection, such as chemiluminescence (Liang et al., 2014; Xu et al., 2010), electrochemical (Yin et al., 2015c, 2015d; Zhang et al., 2015), fluorescent (Deng et al., 2015; Qian et al., 2015b; Wang et al., 2015b) and surface-enhanced Raman spectroscopy (Liu et al., 2014b). Among these methods, electrochemistry has the advantages of relatively simple instrumentation, high sensitivity and mild operating conditions (Kerman et al., 2008; Luo et al., 2016; Miao et al., 2012). However, to the best of our knowledge, no study has reported the detection of both PKA and ALP using a single electrochemical biosensor.

Herein, we report an electrochemical biosensor for evaluating the activity and inhibition of both PKA and ALP. PKA can hydrolysis ATP to adenosine diphosphate (ADP), while ALP can hydrolysis pyrophosphate (PPi). These two processes resulted in the production of one common byproduct, phosphate ions (Pi). The following reaction of Pi with acidic molybdate could form a molybdophosphate precipitate on the electrode surface, generating electrochemical current that is proportional to the activity of PKA or ALP (Talarico et al., 2015; Xie et al., 2015). The inhibition effect of H-89 on the activity of PKA and NaF on the activity of ALP were also studied as well as the feasibility of the biosensor for the detection of PKA in HeLa cell lysates. The developed biosensor thus holds great promise for clinical application and biomedical research.

2. Experimental methods

2.1. Materials and apparatus

Cyclic adenosine 3′,5′-monophosphate (cAMP)-dependent protein kinase (PKA) was purchased from New England Biolabs Inc. (Beverly, MA). Adenosine-5′-triphosphate (ATP) was obtained from Generay Biotech Co., Ltd. (Shanghai, China). Adenosine-5′-diphosphate disodium salt ($ADPNa_2$), forskolin and H-89 were acquired from Beyotime Institute of Biotechnology (Shanghai, China). 3-isobutyl-1-methylxanthine (IBMX), sodium molybdate dihydrate ($Na_2MoO_4 \cdot 2H_2O$), alkaline phosphatase (ALP), sodium pyrophosphate decahydrate ($Na_4P_2O_7 10\ H_2O$, PPi), glucose oxidase (GOx), alcohol dehydrogenase (ADH) and β-Site Amyloid Precursor Protein Cleaving Enzyme 1 (BACE1) were obtained from Sigma-Aldrich. Glutathione(GSH) was received from Energy Chemical (Shanghai, China). Human serum albumin (HSA) was purchased from Zhenglong Biochem. Lab (Chengdu, China). Sodium fluoride (NaF) was obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). 10 mM Tris–HCl buffer (pH = 7.4, 100 mM NaCl) was used as the buffer solution. Other reagents were of analytical grade and used without further purification. All stock solutions were prepared with double-distilled water.

Electrochemical measurements were performed on a CHI-650D electrochemical workstation (Shanghai CH Instruments Co., China). A conventional three-electrode system was used with a gold electrode (Au, 2 mm in diameter) as the working electrode, an Ag/AgCl electrode as the reference electrode and a platinum wire as the auxiliary electrode. For testing PKA activity in cell lysates, the cells were broken with Fisher Model FB120 ultrasonic processor.

2.2. Electrochemical characterization of the molybdophosphate precipitate

For the modification of the electrode, 5 μL of graphene oxide (GO) solution (20 mg/mL) was dropped on the Au electrode surface. After dried, 15 μL of the solution containing 200 μM of Na_2HPO_4 and 6 mM of Na_2MoO_4 solution were dropped onto Au electrode surface for 20 min and then tested.

2.3. Electrochemical detecting of PKA activity and inhibition

To test the reaction of ATP with Na_2MoO_4, a specific concentration of ATP solution was reacted with 6 mM Na_2MoO_4 on GO modified Au electrode according to above mentioned procedure. For PKA test, different concentrations of PKA were mixed with 250 μM of ATP and incubated at 37 °C for 1 h. The resulting solution was then mixed with 6 mM Na_2MoO_4 on the electrode and then tested.

For assay of PKA inhibition, 50 U/mL of PKA was initially incubated with various concentrations of protein kinase inhibitor H-89 for 1 h, and then the activity of PKA was measured.

2.4. Electrochemical detecting of ALP activity and inhibition

For measurement of ALP activity, different concentrations of ALP were mixed with 100 μM PPi and incubated at 37 °C for 1 h. The resulted solution was then mixed with 6 mM of Na_2MoO_4 for 20 min and tested.

For assay of ALP inhibition, 200 U/L of ALP was incubated with the inhibitor NaF of various concentrations for 1 h. Then, the activity of ALP was measured.

2.5. Performance of the sensor for testing PKA activity in Hela cell lysates

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 (1:1) supplemented with 10% fetal bovine serum. The cells were incubated under a humidified atmosphere containing 5% CO_2 at 37 °C in a cell culture box. The culture medium was then replaced by serum-free medium (pH = 7.4, Tris–HCl,10 mM NaCl) before stimulation. Various concentrations of forskolin and IBMX were added into the medium to activate the intracellular PKA for 30 min For comparison, 400 μL of Tris–HCl was added into the medium as a control, and 4 μM H-89 was added into one sample as another control. The cells (3.75×10^5/mL) were broken with an ultrasonic processor for 40 min, which was performed at 0 °C with ultrasonic interval time of 5 s The ultrasonic frequency was 20 KHz. The cell lysates were centrifuged at 2000 r/min, and the supernatant was ready for test.

3. Results and discussion

3.1. Electrochemical characterization of the molybdophosphate precipitate

The basic concept in this work is to develop an electrochemical sensor for monitoring the activity of both PKA and ALP based on phosphate ions induced deposition of redox precipitates. Fig. 1 addresses the schematic representation of the sensing principle. Initially, the reaction of phosphate ions with molybdate was studied. Graphene oxide (GO) was utilized to modify the gold electrode since the good conductivity of GO could enhance the electrochemical current of redox species (Brownson et al., 2015, 2014). GO was synthesized from natural graphite by Hummers' method according to literature reports (Dikin et al., 2007; Sun et al., 2013).