Analytical Biochemistry 441 (2013) 185–189

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Real-time monitoring of intracellular signal transduction in PC12 cells by two-dimensional surface plasmon resonance imager

Hiroaki Shinohara [a,b,*], Yhutarou Sakai [a], Tanveer Ahamd Mir [b,*]

[a] Field of Life, Information and System Sciences, Graduate School of Science and Engineering for Research, University of Toyama, Toyama 930-8555, Japan
[b] Major of Biological Information Systems Science, Graduate School of Innovative Life Science for Education, University of Toyama, Toyama 930-8555, Japan

ARTICLE INFO

Article history:
Received 1 June 2013
Received in revised form 10 July 2013
Accepted 13 July 2013
Available online 23 July 2013

Keywords:
2D–SPR imaging
PC12 cell
Signal transduction

ABSTRACT

Real-time observation of intracellular process of signal transduction is very useful for biomedical and pharmaceutical applications as well as for basic research work of cell biology. The conventional methods used to observe intracellular reactions have not been convenient with several steps such as labeling and washing steps prior to the readout. Consequently, there is a critical need for label-free observation techniques for monitoring intracellular reactions. For feasible and reagentless observation of intracellular alterations in real time, we examined the use of a high-resolution two-dimensional surface plasmon resonance (2D–SPR) imager for monitoring of intracellular signal transduction that was mainly translocation of protein kinase C via local refractive index change in PC12 cells adhered on a gold sensor slide without any indicator reagent. PC12 cells were stimulated with KCl and phorbol-12-myristate-13-acetate (PMA, a protein kinase C [PKC] activator) at different concentrations in order to induce intracellular PKC translocation. 2D–SPR signal (reflection intensity change) is very consistent with the cellular response normally detected for these stimulants. Our results suggest that complex intracellular reactions could be real-time monitored and characterized by the 2D–SPR imager. It is further expected that signal transmission that was followed by the translocation of signaling proteins could be observed at the single cell level with the high-resolution 2D–SPR imager.

Protein kinase C (PKC)[1] is a family of Ser/Thr kinases that play an essential role in a multitude of cellular processes, including control of fundamental autonomous activities such as cell survival, proliferation, and differentiation [1]. In the nervous system, PKC has been linked to the regulation of axonal and dendritic growth, neural cell adhesion molecule-mediated neurite outgrowth [2], neurotransmission [3], and axonal regeneration [4]. So far, significant progress has been made through studies based on analysis of translocation of PKC activity from cytosolic to membrane fractions of intact cells by techniques such as immunoblotting [5] and fractionation of cytosol and membrane fraction and vice versa [6]. Accordingly, in addition to elucidation of signal transduction mechanisms that control fundamental cellular processes, accurate noninvasive and real-time observation of intracellular PKC activity is expected to provide insights regarding the underlying basis of disorders.

Recent technical developments enabled us to monitor the movement of PKC in living cells using PKC fused with green fluorescent protein (GFP) [7–11]. However, all of these breakthrough experiments are limited by the following factors: required genetically engineered labeling of the target molecule and complicated and tedious optimization steps that can affect the signal, throughput, time, and utility of the assay [12].

Hence, there is a need for an improved assay method that addresses most of these limitations and concurrently provides an additional ability to assess a compound's toxicity, specificity, selectivity, potency, and effectiveness within a cellular context in a label-free format.

In this respect, a two-dimensional surface plasmon resonance (2D–SPR) imager fulfills the requirements for a nondestructive, label-free assay of nearly all living cells and tissues and can be used for sensitive measurements attributed to changes in refractive index or the dielectric constant near the surface of a thin metal layer using the SPR phenomenon [13,14]. Conventional one-dimensional SPR sensing, following cell stimulation, was previously applied to the monitoring of mast cells stimulated by antigens and was proposed to reflect the assembly of molecules near the plasma membrane [15,16]. Moreover, the conventional 2D–SPR biosensor technique has also been implemented for living cells in order to

* Corresponding authors. at: Biological Information Systems Science, Graduate School of Innovative Life Science for Education, University of Toyama, Toyama 930-8555, Japan. Fax: +81 076 445 6832.

E-mail addresses: hshinoha@eng.u-toyama.ac.jp (H. Shinohara), mirtanveer0699@gmail.com (T.A. Mir).

[1] *Abbreviations used:* PKC, protein kinase C; GFP, green fluorescent protein; 2D–SPR, two-dimensional surface plasmon resonance; DMEM, Dulbecco's modified Eagle's medium; PMA, phorbol-12-myristate-13-acetate; CCD, charge-coupled device.

0003-2697/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ab.2013.07.017

monitor morphological changes using antibodies [17,18]. However, the magnification of the conventional 2D–SPR imager is not sufficiently high to monitor cellular response at the single cell level. To address this drawback, we previously reported that a high-resolution 2D–SPR imager was available for real-time observation of allergen responses of a mast model cell, rat basophilic leukemia cell (RBL–2H3) and found, furthermore, that the high-resolution 2D–SPR imager was capable of monitoring cellular response at the individual cell level [19,20].

In this study, we combined a high-resolution 2D–SPR biosensor platform with PC12 cells, a model nerve precursor cell for real-time monitoring of intracellular signal transduction that was mainly translocation of PKC on chemical stimulation. Experimental data confirmed that a rapid and transient enhancement in PKC translocation in individual PC12 cells occurred by depolarization stimulation with a high concentration of K^+ ions. Our results indicated that the high-resolution 2D–SPR imager could allow us the spatiotemporal investigations of signal molecular recruiting to and dissociating from the cell membrane dynamically triggered by external stimuli.

Materials and methods

Chemicals and materials

The chemicals and cells were obtained from the following sources. PC12 cells (RCB0009) were obtained from the cell bank of RIKEN BioResource Center. Dulbecco's modified Eagle's medium (DMEM), penicillin–streptomycin, and horse serum were purchased from Gibco, and fetal bovine serum was obtained from ICN Biomedicals. Phorbol-12-myristate-13-acetate (PMA), staurosporine, and modified Hanks' balanced salt powder were purchased from Sigma. KCl was obtained from Wako Pure Chemical Industries. In addition, 50-nm gold layer deposited high-refractive index glass (SF6) chips (18 × 17 mm) were purchased from BAS (Japan), and flexiPERM (11 × 7 × 10 mm) was purchased from Greiner Bio One.

Cell culture

PC12 cells were grown on culture flasks (25 cm^2), with DMEM supplemented with 10% (v/v) horse serum (Gibco), 5% (v/v) fetal bovine serum (ICN Biomedicals), and 1% penicillin–streptomycin (Gibco). The cells were maintained in an incubator at 37 °C in 5% CO_2.

Preparation of SPR sensor slide

Sensor slide was prepared by using a gold chip walled with a piece of square well (11 × 7 × 10 mm)-type flexiPERM. Prior to cell culture, SPR sensor slides were put into small cell culture dishes and preincubated with ultraviolet (UV) sterilization for 10 min. PC12 cells were removed from the bottom of the cell culture flasks by standard trypsinization and suspended in 5 ml of culture medium. The cell suspension was then diluted to 4 × 10^5 cells/ml with culture medium, and 300 μl of cell suspension was poured into the gold sensor slide placed in culture dishes. The sensor slide containing cells was allowed to incubate for 24 h at 37 °C in 5% CO_2 before being placed on the 2D–SPR device station for measurement of refractive index change. The cells were allowed to attach and spread typically for 24 h to reach a stable baseline before the chemical stimulation.

Experimental setup for SPR imaging

2D–SPR experiments were performed with a high-resolution 2D–SPR apparatus (2D–SPR 04A, NTT Advanced Technology [NTT–AT], Japan) possessing a collimator, four magnification lenses (1×, 2×, 4×, and 7×), and a cooled charge-coupled device (CCD) camera, as shown in Fig. 1 The 2D–SPR images of PC12 cell areas were obtained by using the 7× magnification lens. Just before the experiment, the cells adhered on the sensor slide were observed directly by phase contrast microscopy. The culture medium was then removed from the sensor slide, and cells were washed twice with Hanks' solution (pH 7.4, 37 °C). After washing, Hanks' solution (240 μl) was added. The gold sensor slide, on which the PC12 cells were cultured, was placed on the top of the prism, and an index matching fluid was used between the prism and the SPR chip in order to eliminate the unwanted reflection. Then 30 μl of Hanks' solution, KCl, or PMA solution as a stimulant solution was gently injected by a manual pipette to the sensor slides, and SPR signals that were reflection intensity change or SPR curves were recorded and analyzed on a computer. For PKC inhibition, cells were pretreated with 100 nM staurosporine (a PKC inhibitor) solution for 30 min prior to PMA stimulation. Hanks' solution (30 μl) was injected for a negative control experiment.

Results and discussion

2D–SPR monitoring of intracellular signal transduction in PC12 cells stimulated with high KCl

The 2D–SPR imager is an evanescent wave-based sensor and is capable of imaging local refractive index changes in the vicinity of the sensor surface [13]. To study PKC signaling, we applied the high-resolution 2D–SPR imager for real-time monitoring of intracellular signal transduction that was mainly PKC translocation in living PC12 cells. PC12 cells were stimulated with different concentrations of K^+ solution to depolarize the cells. The cell regions on the gold sensor chip are well identified separately (Fig. 2) and images are pictured by the CCD camera. To rule out possible confounding effects of the measurement buffer solution that may stem from the mechanical strain induced by the turbulence during solution ejection by manual pipette, cells were tested with Hanks' solution for negative control. Injection of Hanks' solution onto these cells produced no detectable change of reflection intensity. Fig. 2A and B show 2D–SPR images of PC12 cell regions before and after stimulation of 100 mM K^+ solution. A dramatic increase of reflection intensity was observed at almost all cell regions. Fig. 2C shows the time course of reflection intensities at five cell regions. As can be seen in Fig. 2C, the reflection intensity of the PC12 cell region stood up so rapidly and individual cell regions showed a similar response pattern; however, the reflection intensity was uneven. This pattern of reflection intensity increase was very similar to the PKC translocation pattern in PC12 cells as reported by Fountainhas and coworkers in 2005 [21]. The excess cell-to-cell

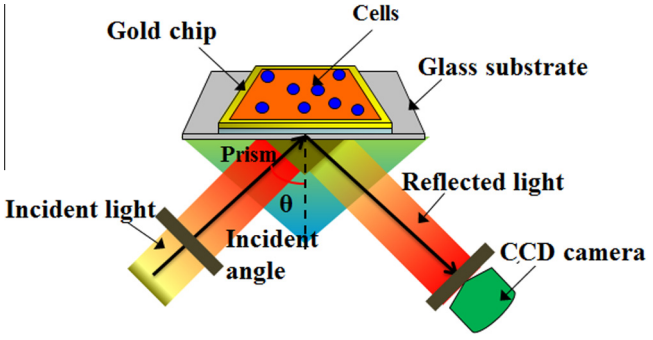

Fig.1. Schematic illustration of the used 2D–SPR system equipped with collimator and a CCD camera with four kinds of lens (1×, 2×, 4×, and 7×) for image magnification.