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Chemical fixation to arrest phospholipid signaling for chemical cytometry

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ABSTRACT

Chemical cytometry is a powerful tool for measuring biological processes such as enzymatic signaling at the single cell level. Among these technologies, single-cell capillary zone electrophoresis (CZE) has emerged as a powerful tool to assay a wide range of cellular metabolites. However, analysis of dynamic processes within cells remains challenging as signaling pathways are rapidly altered in response to changes in the cellular environment, including cell manipulation and storage. To address these limitations, we describe a method for chemical fixation of cells to stop the cellular reactions to preserve the integrity of key signaling molecules or reporters within the cell and to enable the cell to act as a storage reservoir for the reporter and its metabolites prior to assay by single-cell CZE. Fluorescent phosphatidylinositol 4,5-bisphosphate reporters were loaded into cells and the cells were chemically fixed and stored prior to analysis. The reporter and its metabolites were electrophoretically separated by single-cell CZE. Chemical fixation parameters such as fixative, fixation time, storage solution, storage duration, and extraction solution were optimized. When cells were loaded with a fluorescent C6- or C16-PIP_2 followed by glutaraldehyde fixation and immediate analysis, $24 \pm 2\%$ and $139 \pm 12\%$ of the lipid was recoverable, respectively, when compared to an unfixed control. Storage of the cells for 24 h yielded recoverable lipid of $61 \pm 3\%$ (C6-PIP_2) and $55 \pm 5\%$ (C16-PIP_2) when compared to cells analyzed immediately after fixation. The metabolites observed with and without fixation were identical. Measurement of phospholipase C activity in single leukemic cells in response to an agonist demonstrated the capability of chemical fixation coupled to single-cell CZE to yield an accurate snapshot of cellular reactions with the probe. This methodology enables cell assay with the reporter to be separated in space and time from reporter metabolite quantification while preserving assay integrity.

1. Introduction

Phospholipids are crucial components in all living cells, serving as second messengers for signal transduction and as structural components for cell membranes [1,2]. Several lipid metabolic pathways exist in cells regulating cellular biology, although measuring the ebb and flow of information within these pathways has proven a challenge due to difficulties in analyzing lipid metabolites in single cells. One example is the G-protein coupled phospholipase C (PLC) pathway which serves a critical role in production of the lipid second messengers diacylglycerol (DAG) and inositol trisphosphate (IP_3) by metabolism of phosphatidylinositol (4,5)-bisphosphate (PI[4,5]P_2) [3,4]. This pathway acts to regulate multiple cellular functions, such as proliferation, mitogenic signal transduction, and response to external stimuli *via* its influence on downsteam effectors, such as protein kinase C and intracellular calcium [1,5,6]. Moreover, multiple other metabolites upstream and downstream of PI(4,5)P_2 are known to be of importance in cellular function, but studies have been limited by a lack of appropriate technologies to make measurements in individual cells. By virtue of their diverse roles in cellular biology and dysregulation in disease states, measurement of the activity of lipid metabolizing enzymes, including PLC, would be of great utility in understanding the molecular mechanisms driving many cellular behaviors.

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However, measurement of metabolites within the PLC pathway has proven very challenging because of their relatively low abundance in cells, the polar nature of the lipids, and the highly acidic head group [7]. Traditionally, radiolabeling of lipids followed by either thin-layer chromatography (TLC) or high performance liquid chromatography (HPLC) has been used [8,9], but both TLC and HPLC suffer from low resolution, require large numbers of cells, and are of low sensitivity. Mass spectrometry has been used to monitor various lipid metabolites [7], but suffers from limited applicability for primary cells because of the need to derivatize these lipids and an inability to distinguish between isomers that are prevalent in phospholipid metabolism. To best quantify the low concentrations of PI(4,5)P_2 lipids and metabolites, an analytical technique capable of isomer resolution with the sensitivity to detect very low levels of lipid, preferably at the single cell level, is required.

Chemical cytometry uses highly sensitive analytical techniques which are ideally suited for measuring small quantities of analytes in single cells, such as single-cell capillary zone electrophoresis (CZE). Single-cell CZE was pioneered by Jorgenson and Kennedy in the early '80s and has enabled unique and powerful assays of the metabolic processes of single cells [10]. Single-cell CZE is often coupled with laser-induced fluorescence detection (CZE-LIF) for ultra-low limits of detection of cellular components [11,12]. The benefits of single-cell analysis have become increasingly apparent in recent years as heterogeneity among cells in a population has been identified [13–15]. A variety of cellular metabolites have been assayed using single-cell CZE, including thiol-containing compounds [16], small molecules such as glucose [17], dopamine [18], and amino acids [19], as well as measures of enzyme and peptidase activity [20–23]. Single-cell CZE has also been used to study different classes of lipid metabolism in cytosolic lysates and single cells [24], such as sphingolipids [25,26], glycosphingolipids [27], and phosphatidylinositides [28].

A difficulty inherent to chemical cytometry methods is that analyzing dynamic metabolic processes can be extremely challenging [29,30]. Cells are sensitive to small changes in the environment, such as temperature, salt concentration, or pH. Signaling pathways can be rapidly altered as a cell copes with a stressor [31]; thus, cytometry techniques must not perturb the cells prior to sampling or artifacts can be generated in the measurement. This can be problematic when the cells originate from a biopsy or surgical specimen and are transported to a distant site to conduct the assay. A method for halting cellular reactions in all cells simultaneously would be of great utility for chemical cytometry measurements of single cells. The Dovichi group pioneered the use of formalin fixation for analysis of glycosphingolipids for cell preservation prior to single-cell CZE [32–35]. When exposed to fixative, amino groups of proteins are amine-crosslinked, which terminates cellular reactions [36–39]. Depending on their size, molecules that are not physically cross-linked can either be entrapped within the cross-linked framework or diffuse out of the cell. Lipids without amino groups, such as the phosphatidylinositide lipids, are not cross-linked by common fixatives but are frequently retained within the cross-linked framework, leaving them accessible for interrogation by an appropriate analysis technique [40–43].

A goal of this work was to explore whether chemical fixation might be coupled to assay of phospholipid metabolism in single cells followed by single-cell CZE. Multiple fixatives were assayed for their impact on the phospholipid probe retention and metabolism. Parameters optimized include the fixation time, extraction solution, and cell storage solution time prior to the CZE assay. Electrophoretic separation buffers capable of resolving hydrophobic phosphatidylinositides from fixed cells were evaluated. The ionophore ionomycin increases free intracellular calcium which stimulates PLC activity. The metabolism of PI(4,5)P_2 to DAG by PLC was characterized in basal and agonist-stimulated cells, both fixed and unfixed. Lastly, formation of DAG was characterized in stored, fixed, single cells via single-cell CZE revealing the heterogeneity in PLC activity in a population of cells.

2. Material and methods

2.1. Materials

Bodipy-labeled phosphatidylinositol 4,5-bisphosphate with a 6-carbon or 16-carbon acyl chain (C6-PIP_2 or C16-PIP_2), bodipy-labeled phosphatidylinositol 3,4,5-trisphosphate with a 6-carbon or 16-carbon acyl chain (C6-PIP_3 or C16-PIP_3, chemical structures shown in Supplemental Fig. S1), H1 histone protein, active SHIP2 lipid phosphatase, and SHIP2 reaction buffer were purchased from Echelon Biosciences (Salt Lake City, UT). Roswell Park Memorial Institute media (RPMI-1640) was procured from Cellgro (Manassas, VA). Fetal bovine serum (FBS) was bought from Atlanta Biologicals (Flowery Branch, GA). Penicillin/streptomycin was purchased from Gibco (Grand Island, NY). Glutaraldehyde was obtained from Sigma-Aldrich (St. Louis, MO). Formaldehyde was purchased from ThermoScientific (Waltham, MA). The glyoxal-containing fixative Prefer was procured from Anatech Ltd (Battle Creek, MI). Ionomycin was obtained from EMD Millipore (Billerica, MA). All other chemicals were purchased from Fisher or Sigma.

2.2. Cell culture

K-562 cells (human chronic myelogenous leukemia lymphoblasts) were obtained from the American Type Culture Collection (ATCC) [44]. Cells were propagated in RPMI-1640 media supplemented with 10% FBS, penicillin (100 units mL^{-1}), and streptomycin (100 $\mu g\,mL^{-1}$) and were maintained in a humidified atmosphere of 37 °C in 5% CO_2 and passaged into fresh media every 3–4 days. Cells were not used beyond passage #10 from the original ATCC stock.

2.3. Generation of C16-PI(3,4)P_2 standard

C16-acyl chain phosphatidylinositol 3,4-bisphosphate, C16-PI(3,4)P_2, was generated by reaction of 10 μM C16-PIP_3 with 78 nM active SHIP2 phosphatase in SHIP2 reaction buffer for 60 min. Complete conversion from C16-PIP_3 to C16-PI(3,4)P_2 was verified with capillary electrophoresis.

2.4. Capillary zone electrophoresis for separation buffer optimization

For the separation buffer optimization experiments, the background electrolyte utilized was NaH_2PO_4, with other components modified as indicated. CZE-LIF was performed on a ProteomeLab PA800 (Beckman Coulter; Brea, CA). Fused silica capillaries [30 μm inner diameter and 360 μm outer diameter (Polymicro Technologies; Phoenix, AZ)] were 30 cm long with a 20 cm effective length. Capillaries were conditioned prior to use by rinsing for 1 h in deionized H_2O, 12 h in 0.1 M NaOH, 1 h in H_2O, 6 h in 0.1 M HCl, and 12 h in H_2O. Prior to each run, the capillary was rinsed with 1 M NaOH, H_2O, and separation buffer for 2 min each by application of 20 psi to the capillary inlet. The standard sample containing C16-PIP_2, C16-PI(3,4)P_2, and C16-PIP_3 was hydrodynamically injected for 5 s by application of 0.5 psi to the capillary inlet and electrophoretic separation was initiated by application of a negative voltage to the outlet electrode while holding the inlet at ground. Field strength varied for each separation buffer sampled; the working voltage was determined by generation of an Ohm's plot with the highest voltage before Joule heating was observed selected as the operating voltage for each separation buffer. Each experiment

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