Chapter 14

Imaging Oscillations of Protein Kinase C Activity in Cells

Maya T. Kunkel and Alexandra C. Newton

Abstract

The protein kinase C (PKC) family contains ten members that share a catalytic core but differ in the composition of their regulatory modules. The conventional PKCs are a subfamily whose regulatory domains bind to Ca^{2+} and to the lipid second messenger diacylglycerol and thus they are activated by coincidence detection of Ca^{2+} and diacylglycerol at the plasma membrane. In HeLa cells, oscillations in Ca^{2+} evoke oscillations in membrane PKC activity. The only method to date that offers the time resolution to observe these rapid changes in PKC activity is the utilization of a genetically encoded membrane-targeted PKC activity reporter, MyrPalm-CKAR (C Kinase Activity Reporter). CKAR is a fluorescence resonance energy transfer (FRET)-based, modular protein that undergoes a conformational change upon phosphorylation resulting in a change in FRET, thereby serving as a direct readout of cellular kinase activity. As a genetically encoded reporter, it is easily introduced into cells and can be targeted to distinct intracellular compartments through the addition of short targeting sequences. MyrPalm-CKAR is targeted to plasma membranes through the amino-terminal addition of seven amino acids that encode a sequence that is myristoylated and palmitoylated in the cell. This chapter details the method of utilizing MyrPalm-CKAR to monitor acute changes in PKC signaling at the plasma membrane that are a consequence of acute changes in Ca^{2+} levels.

Key words: PKC, FRET, Kinase activity reporter, CKAR, Phorbol esters, Diacylglycerol, Ca^{2+}, Live cell imaging

1. Background and Historical Overview

The PKC family of isozymes is perched on a branch of the kinase tree that contains the AGC kinases (1). The ten members of the PKC family have in common a conserved catalytic core but differ in the composition of their regulatory modules (reviewed in (2)). It is these differences in their regulatory modules that control their regulation by lipid second messengers and Ca^{2+}. Conventional isozymes (PKC α, β, and γ) are regulated by Ca^{2+} via a C2 domain and by diacylglycerol via tandem C1 domains; novel isozymes (PKC δ, ε, θ, η) are regulated by diacylglycerol, and atypical PKC

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isozymes (ζ, ι/λ) are regulated by scaffold interactions via a PB1 domain.

PKC isozymes transduce an abundance of signals and play central, and sometimes opposing, roles in cell survival and apoptotic pathways (2, 3). Misregulation of PKC leads to pathogenic states, notably cancer (4). PKC isozymes are highly expressed in the brain, where an abundance of substrates have been identified (5). Expression of the conventional isozyme PKC γ and an alternatively transcribed form of the atypical PKC ζ consisting only of its catalytic domain, PKM ζ, are confined to brain, suggesting a unique role of PKC signaling in brain (6, 7). A number of naturally occurring mutations in PKC γ have been identified in patients with spinocerebellar ataxia type 14, resulting in reduced protein stability and signaling output (8, 9). PKM ζ has been proposed to play a key role in learning and memory (10), although this work remains controversial because experiments to date have depended on the use of pharmacological inhibitors that do not inhibit PKM ζ in cells (11). Another important isozyme in the brain is PKC α, an isozyme that is required for long term depression via a mechanism that depends on scaffolding via its C-terminal PDZ ligand (12).

The advent of genetically encoded kinase activity reporters has provided previously unattainable insight into the spatiotemporal dynamics of PKC activity in cells (13, 14). Targeting the reporter to discrete intracellular locations by fusion of appropriate targeting sequences has allowed visualization of the rate, amplitude, and duration of PKC signaling at the plasma membrane, Golgi membranes, mitochondria, cytosol, and nucleus (15). Furthermore, the generation of an isozyme-specific reporter, δCKAR, has enabled dissection of the role of PKCδ in second messenger signaling (16).

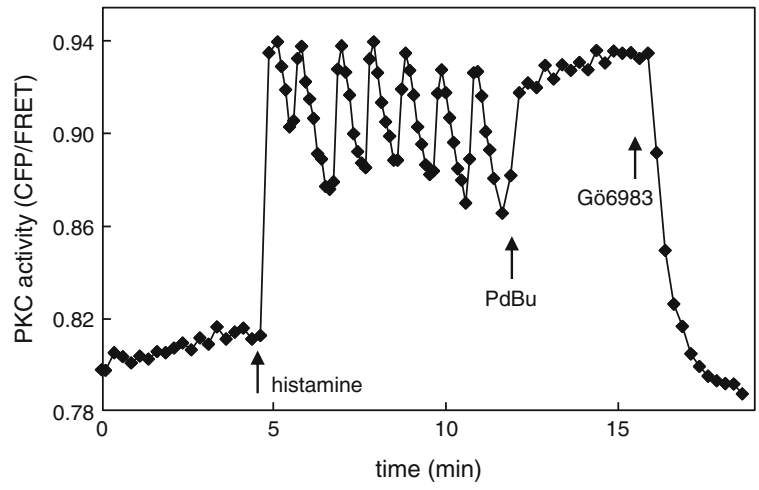

Fig. 1. The CFP/FRET ratio from MyrPalm-CKAR oscillates after stimulation of HeLa cells with 10 μM histamine. Subsequent addition of 200 nM phorbol-12,13-dibutyrate (PdBu) maximally activates PKCs and the FRET change from CKAR. The FRET ratio from CKAR reverses once PKCs are inhibited by addition of the PKC inhibitor Gö6983.

The ability to monitor PKC activity at specific cellular locations has revealed a unique signature of activity depending on cellular location. Relevant to this chapter, the activity of conventional PKC isozymes dominates at the plasma membrane, where under appropriate conditions it oscillates in phase with Ca^{2+} oscillations (see Fig. 1) (13). Here, we describe how to measure oscillatory PKC activity in response to agonist stimulation.

2. Materials

1. Sterile glass-bottom culture dishes (MatTek Corporation)
2. HeLa cells
3. FuGENE 6 transfection reagent (Roche)
4. DNA encoding MyrPalm-CKAR (distributed by Addgene)
5. Hanks' Balanced Salt Solution (HBSS) containing 1 mM Ca^{2+}
6. Axiovert 200 M microscope (Zeiss)
7. MicroMAX 512BFT CCD camera (Roper Scientific)
8. Lambda 10-2 filterwheel shutter controller (Sutter)
9. Metafluor software (Molecular Devices)
10. 40×/1.3 NA oil-immersion objective (Zeiss)
11. Immersion oil, Type DF (Cargille Labs)
12. Filters from Chroma Technology:
    (a) CFP: 420/20 nm excitation, 450 nm dichroic, 475/40 nm emission
    (b) YFP: 495/10 nm excitation, 505 nm dichroic, 535/25 nm emission
    (c) 10% neutral density filter
13. histamine (Sigma)
14. phorbol-12,13-dibutyrate (Calbiochem)
15. Gö6983 (Calbiochem)
16. Excel (Microsoft)

3. Methods

Day 1:

Plate HeLa cells on a sterile glass-bottomed tissue culture dish.

Day 2:

Transfect HeLa cells that are between 50 and 80% confluent with 1 μg of MyrPalm-CKAR DNA using FuGENE 6 according to the manufacturer's protocol.

Day 3:

1. Turn on the microscope, camera, filterwheel controller and lamp.
2. Launch Metafluor software and allow CCD camera to cool (approximately 15 min).
3. Remove media from the cells to prepare the dish for the imaging experiment.
4. Rinse cells one time in HBSS, image in 2 ml HBSS.
5. Clean the 40× oil-immersion objective and apply one drop of oil.
6. Place the imaging dish on the microscope stage and secure it (we use clay) to prevent subtle movements of the dish during the addition of agonists/inhibitors over the course of the experiment.
7. Focus on the cells.
8. The following details were established on the above setup to allow for FRET imaging of CFP- and YFP-based FRET experiments:
   (a) Acquire all fluorescent images through a 10% neutral density filter.
   (b) Perform all experiments at room temperature.
   (c) Set up a protocol within Metafluor to acquire CFP, FRET, YFP, and CFP/FRET with the parameters listed below.
      - Acquire CFP for 200 ms using 420/20 nm excitation, 450 nm dichroic, 475/40 nm emission filters.
      - Acquire FRET for 200 ms using 420/20 nm excitation, 450 nm dichroic, 535/25 nm emission filters.
      - Acquire YFP for 100 ms using 495/10 nm excitation, 505 nm dichroic, 535/25 nm emission filters (this channel is acquired to monitor against photobleaching of the FRET acceptor).
      - Find cells expressing approximately 1 μM of MyrPalm-CKAR (too much or too little will not yield optimal results). In our setup, this was calibrated to be between 400 and 800 intensity units in the CFP channel and 1,000–2,000 units in the YFP channel.
   (d) With the above parameters, one can acquire a series of images every 7 s without photobleaching on this setup.
9. Define regions for each cell within the Metafluor software. MyrPalm-CKAR targets the reporter to plasma membranes so we choose a region from the top of the cell and make certain to avoid the edges which may move in or out of the region within the course of the experiment (see Fig. 2).

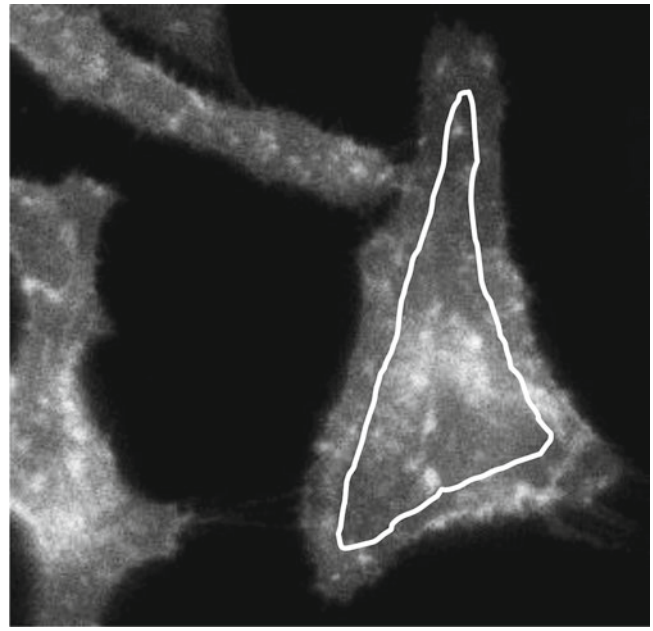

Fig. 2. The fluorescent signal from MyrPalm-CKAR expressed in HeLa cells is distributed throughout plasma membranes of the cells. Shown drawn over the cell image in *white* is the region defined in Metafluor for analysis of FRET ratio changes from MyrPalm-CKAR.

10. Save a log file of the data from within the software.
11. Importantly, save the images within the software as these can be re-analyzed after the experiment if necessary.
12. Begin the experiment acquiring data every 7 s until a constant baseline is established.
13. Once a steady baseline is established, stimulate cells with 10 μM histamine by removing 500 μl of HBSS from the dish, mixing this with the histamine to be added, then adding the mixture back drop-wise to the dish between time points.
14. Monitor the changes in the CFP/FRET ratio following histamine. Oscillations in Ca^{2+} translate to oscillations in membrane PKC activity in HeLa cells; this is observed by oscillations in the CFP/FRET ratio from MyrPalm-CKAR (see Fig. 1).
15. One can add a PKC inhibitor (e.g., Gö6983) to halt/reverse changes in the CFP/FRET ratio from CKAR (Fig. 1).
16. When imaging is complete, close the log file and the saved images file within Metafluor.
17. Data analysis can be performed using an Excel spreadsheet, i.e., open the log file from within Excel to view the data saved. The file will contain time as the first column, followed by four columns from each region analyzed: CFP intensity, FRET intensity, YFP intensity, CFP/FRET ratio calculation.

4. Notes

1. We acquire all of our data at room temperature. The cells are healthy in HBSS for the duration of our experiments (under an hour); furthermore, the lower temperature allows one to visualize rapid changes with better time resolution.
2. Metafluor software is our software of choice for time-lapse FRET experiments, as the software performs a real-time analysis of each region during acquisition. That is, the software quantifies the intensity within the user-defined regions from the CFP, FRET, and YFP channels, and then plots these intensities, as well as the CFP/FRET ratio, after each time point acquired.
3. A CFP/FRET ratio (versus the more common FRET/CFP ratio) is suggested as CKAR displays FRET in the unphosphorylated state and undergoes a decrease in FRET upon phosphorylation by PKC (this is true for all FHA2 domain-based kinase activity reporters such as BKAR and DKAR) (17, 18). Plotting CFP/FRET results in an increase in the ratio when there is an increase in kinase activity.
4. Cells expressing too little or too much reporter will not yield optimal results. Each distinct setup needs to be calibrated for expression levels of reporter. We performed such calibration by determining the approximate concentration of reporter in the cell based on fluorescence intensity and then aimed for imaging approximately 1 μM reporter expressed within a cell (see (19)).
5. Each distinct setup should be tested to determine how much light the reporter can be exposed to (as determined by acquisition times and frequency of acquisition) without photobleaching.
6. If one does not observe changes in the CFP/FRET ratio following histamine stimulation, one can test the setup by adding 200 nM phorbol-12,13-dibutyrate to the cells while imaging; this will robustly stimulate PKC activity at the plasma membrane by binding to its regulatory domains with high affinity.
7. Background levels should be subtracted from each channel post-acquisition in references within Metafluor, after the levels throughout the course of the experiment have been established.

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