Protein kinase C interactions with caveolin determined in intact cells by resonance energy transfer-fluorescence lifetime imaging

C D Stubbs, S W Botchway, A W Parker

Central Laser Facility, CCLRC Rutherford Appleton Laboratory, Chilton, Didcot, Oxon., OX11 0QX, UK

S J Slater

Department of Pathology and Cell Biology, Thomas Jefferson University, Philadelphia PA 19107, USA

Main contact email address: *cdstubbs@rl.ac.uk*

Introduction

In this work the interaction of protein kinase Cα (PKCα) with caveolin, was investigated using two-photon-fluorescence lifetime imaging (2P-FLIM). In common with other signalling proteins, PKC has been suggested to associate with caveolin-1[1,2], a key component of caveoli and membrane rafts, that are proposed to exist as signaling platforms in the plasma membrane and elsewhere in the cell.

The interaction was determined by investigating the fluorescence energy transfer between GFP-tagged PKCα and DsRed-caveolin (DsRed-cav). Using the quenched lifetime of the GFP-tag, areas showing co-localisation in CHO cells were identified after activation of the PKC had occurred. When the GFP-PKC is induced to translocate to membranes, excitation of the GFP at 850 nm, for 2P excitation, should lead to FRET to the DsRed construct, if the two are co-localised. This would be seen as a reduced GFP lifetime, in contrast to areas in which the GFP-PKC residues but not with caveolin. This information was determined using a FLIM time-correlated single photon counting set up (TCSPC) with the frequency doubled output of a Tsunami pulsed laser (100 fs) and coupled to an inverted fluorescence microscope.

Methods

Chinese hamster ovary (CHO) cells were obtained from the ATCC (Manassas, VA) or were generously provided by Dr. Emma Leatherbarrow (MRC, Harwell). Cells were cultured on glass cover slips in F-12 HAM's media (Sigma-Aldrich) supplemented with 10% Foetal calf serum (ICN Biomedicals or Gibco), 1% L-glutamine and 1% penicillin/streptomycin (Sigma Aldrich or Gibco) and cultured until reaching confluence.

For transfection, 1-2 μg of pPKCα -EGFP vector DNA (Clontech) and/or DsRed1-cav-1, (kindly provided by Dr. R. Pagano, Mayo Clinic and Foundation) was added to cells in Lipofectin (Invitrogen), according to the manufacturer's protocol. Cells were incubated for a further 48 h and were then washed with phosphate buffered saline (PBS). Cells were then treated with 4β-12-O-Tetradecanoylphorbol-13-acetate (TPA), calcium ionophore or A23187, (all from Sigma-Aldrich), or were untreated (controls). The cells were then fixed in 3.7% formaldehyde solution in PBS for 20 min at room temperature washed, again with PBS, before mounting on slides with Crystal Mount before FLIM analysis.

Fluorescence lifetime images were obtained using a 2P-microscopy apparatus, using a Nikon TE2000 U microscope, constructed in the Central Laser Facility of the Rutherford Appleton laboratory, which has a Bio-Rad MRC600 confocal scanning system and external x, y galvanometers,[3,4]. Laser light at a wavelength of 850 nm was obtained from a Titanium Sapphire, 82 MHz, mode-locked laser (Spectra-Physics), with a pulse width of 120 fs. The light was focused to a diffraction-limited spot through an air (x40, n.a. 0.9) and specimens illuminated at the microscope stage by passing the beam through the MRC600 scan head or through a dichroic at the epifluorescence port. Fluorescence emission was passed through a BG39 filter (Comar) to remove the laser line. The scan was operated in the normal mode, and line, frame and pixel clock signals were generated and synchronized with an external fast micro channel plate – photomultiplier tube, which was used as the detector (Becker & Hickl, GmbH Germany). These were linked via a TCSPC-PC module SPC700 (Becker & Hickl). The set up was used to excite GFP fluorescence, the lifetime of which was measured as a function of the quenching by DsRed when the GFP-PKC and DsRed-cav were close enough for fluorophore tags to participate in FRET.

Images (6 bit, 256 x 256 pixels) were exported from Becker & Hickl software as bitmaps and converted into TIFF files. Image analysis was performed using either Irfanviewer, FLIM analyses were performed using SPC Image 2.0 software (Becker & Hickl).

Results

The treatment of the cells with the phorbol ester TPA not only induces translocation of PKC to different cell compartments but also produces catalytically active PKC. The various treatments used are summarised in Table 1 and are shown for TPA in Figure 1. In general, PKC moves to the outer membrane and to perinuclear regions, and associates with various signalling and cytostructural components in the cell. The lifetime image data revealed that PKC associates with caveolin, the GFP lifetime being reduced accordingly from ~2.2 ns by close proximity to the DsRed by FRET to 1.5-1.8 ns.

Three discrete areas could be ascertained from visual inspection of the FLIM images. When such areas are relatively small or scattered the software offers a masking option for analysis of that region in isolation or a "single pixel analysis" can be used. Three distinct areas were found at the cell peripheral regions, in the nuclear region and in the perinuclear region (Figure 1b-d), These areas are outlined with dotted white lines. The panel in Figure 1f shows representative *single pixel* analyses for each of the three regions. The lifetimes (single exponential) were 2.0 ns (nuclear), 1.87 ns (peripheral) and 1.48 ns (perinuclear). This indicates that FRET, due to co-localisation of the caveolin and PKC, is predominantly occurring in the perinuclear region (see also analysis of a representative perinuclear *region* (depicted with white arrows in Figure 1e), and to some extent in the peripheral regions of the cell, with little or no co-localisation within the nuclear region, due to a known lack of significant caveolin in that region.

Discussion

In the present study, we show for the first time, using a FRET-FLIM approach, that PKCα and caveolin co-localise upon increased intracellular Ca^{2+} or phorbol ester-induced interaction with PKC, however, in the 'resting' state the two molecules do not interact.

The principle caveolin containing structures in the cell are caveoli, which transport to and from the plasma membrane and endosomes[5]. Here we demonstrated that PKC translocates in a phorbol ester/Ca^{2+}-dependent manner to these regions. To examine this we used a 2-3 min time point, optimal for translocation (result not shown).

We were able to isolate three discrete regions in the cells. These were PKC-GFP in the plasma membrane/peripheral regions; the

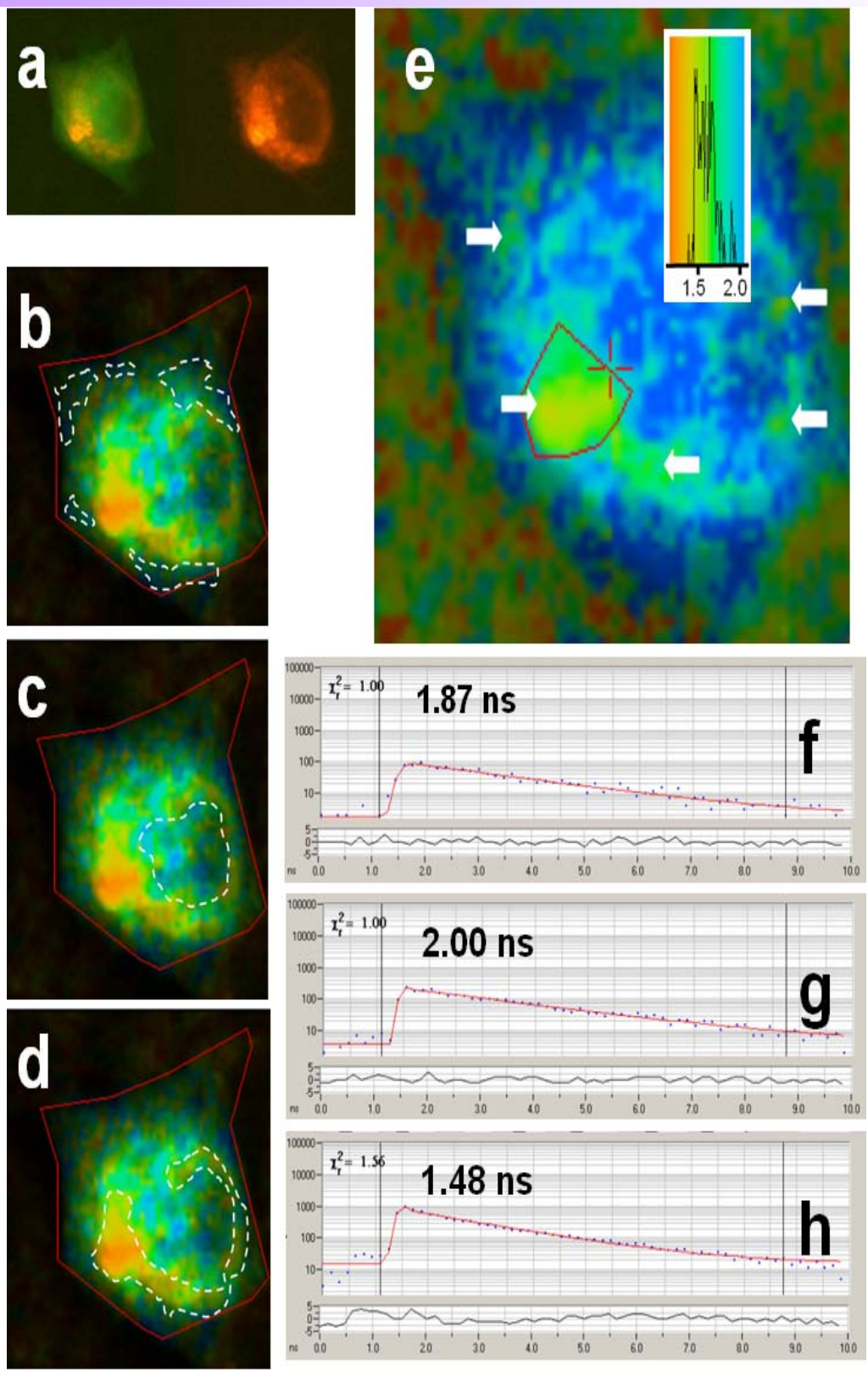

Figure 1. Lifetime imaging of GFP-PKCα co-expressed with DsRed-cav in CHO cells: effect of phorbol ester. PKC-GFP and DsRed-cav were co-expressed in CHO cells over 48 h and then treated with TPA for 2 min before fixation. (a) epifluorescence images of cells with co-expressed GFP-PKC/DsRed-Cav, shown as total red and green fluorescence (left) or (right) red fluorescence (DsRed). In the lifetime images (b), (c) and (d), three distinct areas of PKC-GFP lifetime are discernable, ranging from blue (unquenched GFP) to red (quenched GFP), these areas (bound by white dashes) were also subjected to corresponding single pixel analyses within the areas, as shown in (f), (g), and (h) as follows: (b, f) Peripheral region τ avg:1.8 ns; single point 1.87 ns [χ^2 1.00]); (c, g) the nuclear region (τ avg:2.0 ns; single point 2.00 [χ^2 1.00]); (d, h) the cytoplasm (τ avg:1.5 ns; single point 1.48ns [χ^2 1.56]). (e) shows an example of the derivation of an average lifetime for one the three areas (cytoplasmic): within an area enclosed in red, other similar areas in the cytoplasm are indicated by white arrows (colour coding shown in the inset). Data shown are representative images from replicate experiments.

direct interaction between the GFP and DsRed, attached to the PKC and Caveolin proteins. The peripheral region of the cell also showed a reduced GFP lifetime but by a lesser degree than in the perinuclear region. This could be due to the interaction being indirect but still close enough to allow FRET. Since FRET falls off in intensity according to the inverse forth law it will still have to be close but perhaps with a small intermediate protein. These possibilities remain to be further explored. Finally in this study we chose a point in time, rather than following live cells, since the PKC locality stabilised over a 2-3 min time course and could be conveniently analysed when the cells were fixed and mounted on slides. There is no indication, however, that the signal would be insufficient to follow time-dependent changes as collection times as low as 15 sec. could be used that still allowed a signal sufficient for lifetime analysis.

Table 1. Summary of average lifetimes for GFP emission under various conditions.

Transfection	Treatment	Area analysed	Avg Lifetime (ns)
GFP-PKC	None	cytoplasm	2.2
	None	nucleus	2.15
	TPA	cytoplasm	2.2
	TPA	nucleus	2.3
GFP-PKC/DsRed-cav	None	cytoplasm	2.4
	None	cytoplasm	2.5
	Ca^{2+}	cytoplasm	1.6*
	Ca^{2+}	nucleus	2.0
	TPA	Cytoplasm excluding peripheral	1.5*
	TPA	nucleus	2.0
	TPA	peripheral	1.8*
	Bradykinin	cytoplasm	1.6*

*reduced lifetime indicating PKC-GFP-DsRed-cav interaction

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