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REVIEW

Fluorescence resonance energy transfer imaging of PKC signalling in living cells using genetically encoded fluorescent probes

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Perception of ligands in the extracellular space by transmembrane receptors initiates signal transduction. The conformation change of the receptor induces changes of intracellular signalling components, including altered cellular concentration, altered subcellular location, altered conformation and altered interacting partners. Biochemical approaches have yielded a lot of information about these processes. However, methods that are compatible with analysis of single living cells are often preferred, since cells are highly organized and their response is usually spatially heterogeneous. In addition, the study of signalling cascades requires high temporal resolution. Fluorescence imaging approaches meet these requirements. Moreover, imaging approaches can be combined with genetically encoded green fluorescent protein-based probes that have a high selectivity and sensitivity for the process/molecule of interest. Nowadays, many genetically encoded probes are available for visualizing signalling in living cells. This review is centred on a key regulator of cellular signalling, protein kinase C (PKC). We will discuss imaging approaches that are used for analysing the molecules involved in activation of PKC, visualizing the dynamics of the location of PKC, measuring the conformation of PKC and quantifying the activity of PKC. These approaches are of general interest since they can be applied to study the dynamics, conformation and activity of any protein in living cells.

Keywords: fluorescence resonance energy transfer imaging; protein kinase C signalling; fluorescent probes; G-protein-coupled receptor

1. INTRODUCTION

Cells are continuously perceiving signals that are present in the extracellular space. Correct interpretation of these signals is required for proper functioning of cells and organisms (Meyer & Teruel 2003). When a signalling cascade is modified, e.g. by mutations in genes encoding signalling proteins, the resulting faulty response may have serious consequences and eventually lead to a disease. A key regulator of many cellular processes is protein kinase C (PKC; Newton 2001). Altered PKC activity is associated with diseases such as cancer, diabetes and neurodegeneration. Biochemical approaches have been taken to understand the properties of PKCs, and these studies have yielded a wealth of information. It has become clear from these studies that PKC activity is controlled by a complex interplay of several factors, such as its location, interactions with

lipids, interaction with proteins and its conformation (Liu & Heckman 1998).

Based on homology, three classes of PKC can be discerned, classical PKCs activated by diacylglycerol (DAG) and calcium, novel PKCs activated solely by DAG and atypical PKCs that are not controlled by DAG or calcium (Parker & Murray-Rust 2004). A major pathway activating classical and novel PKCs involves the activation of the enzyme phospholipase C that produces DAG and calcium (Rhee 2001). Besides activation by these second messengers, the classical and novel PKCs can be activated by phorbol ester. This is a compound from plant sap that has tumour-promoting properties (Newton 2004).

A simplified cartoon model shown in figure 1 summarizes the activation of classical PKCs. Nowadays, all of these factors, including its kinase activity, can be measured in single living cells. We will start by discussing the benefits of employing fluorescence microscopy for studying signal transduction. Subsequently, we will discuss how each of these parameters can be quantified

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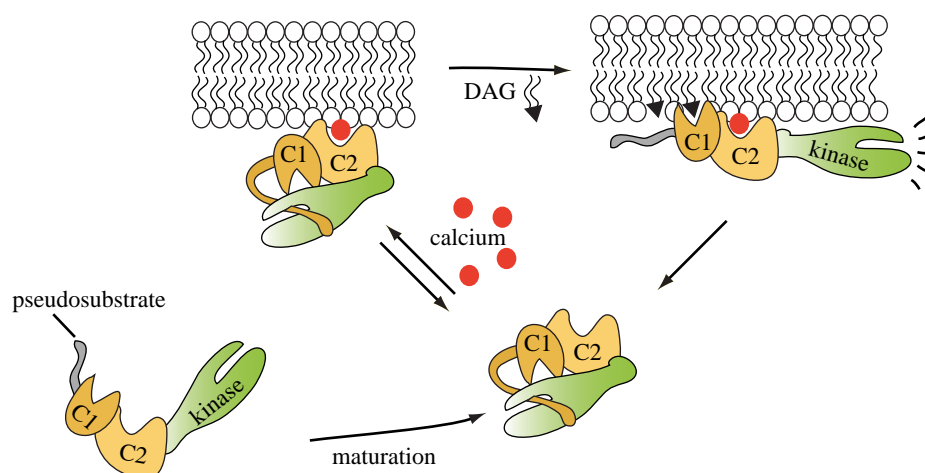


Figure 1. Cartoon model of activation of classical PKC isoforms. Several domains of PKC are depicted; the pseudosubstrate, the DAG-sensitive C1 domain, the calcium-dependent lipid-binding C2 domain and the kinase domain. Before PKC can be activated, it undergoes maturation, requiring phosphorylation by PDK-1 followed by autophosphorylation. The mature PKC is located in the cytoplasm in a closed conformation. The pseudosubstrate occupies the active site, autoinhibiting enzymatic activity. When intracellular calcium levels are increased, PKC can bind the plasma membrane by means of the C2 domain which is a calcium-dependent lipid-binding domain. The C1 domain binds DAG at the plasma membrane, releasing the pseudosubstrate from the active site, thereby relieving the autoinhibition and activating the enzyme. Combined presence of DAG and calcium, e.g. due to activation of PLC, leads to full activation of PKC. The enzyme returns to the cytoplasm when calcium levels are decreased. The phorbol esters bind the C1 domain and induce translocation and activation of the enzyme in the absence of calcium.

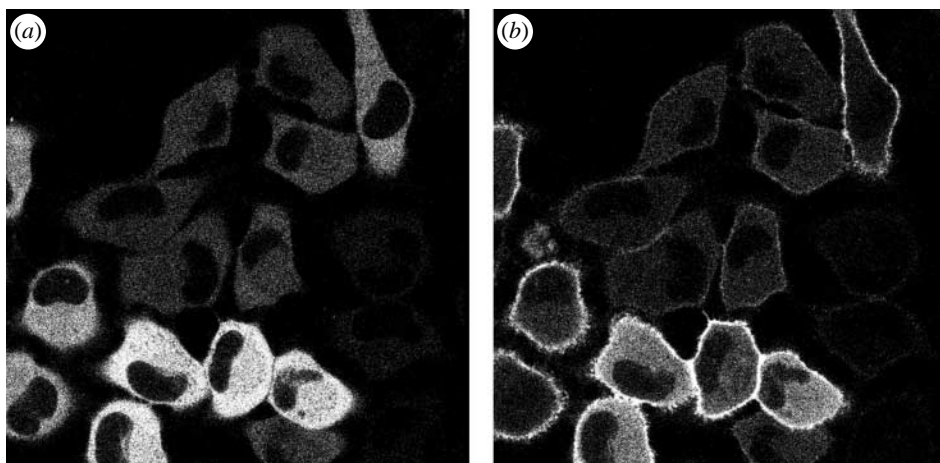


Figure 2. Translocation of PKC α monitored in living cells by confocal microscopy. A GFP-tagged PKC α isoform was expressed in HeLa cells. Single confocal sections through the mid-cell were acquired in time to analyse the distribution of the fluorescently tagged protein in living cells. (a) In resting cells, the protein is located in the cytoplasm and excluded from the nucleus. (b) Addition of 100 nM phorbol ester (PMA) induces the translocation to the plasma membrane. Full width of the images is 146 μ m. Images were acquired using a Zeiss LSM 510 confocal microscope. Excitation was at 488 nm and emission was filtered through a LP505 nm filter and the pinhole was set to 1 Airy unit.

for PKC in single living cells with specific emphasis on fluorescence resonance energy transfer (FRET) and lifetime imaging methodologies. The approaches discussed here will be generally applicable to study dynamic processes in single living cells.

2. WHY USE FLUORESCENCE MICROSCOPY?

There are several good reasons to apply fluorescence microscopy to study cellular processes (Meyer & Teruel 2003; Megason & Fraser 2007). First and most importantly, it is essential to study cellular processes in living, intact cells, with minimal disruption of cellular physiology. Cells are highly organized and often asymmetric in structure, with neurons perhaps being the most prominent example. Hence methods are

required that not only leave the cells intact but also can address the spatial information. Second, cells usually respond heterogeneously, and therefore single-cell experiments are necessary. This is exemplified by cellular processes that exhibit oscillatory patterns, e.g. cell cycle-dependent expression and phosphorylation of proteins, time-dependent expression of proteins involved in circadian rhythms and calcium oscillations in cellular signalling. Since these processes are generally not synchronous between cells, the effects can be revealed only when studying single cells. Third, especially pertinent in signalling studies, many of the changes take place on the millisecond to second time scale, requiring methods that have sufficient temporal resolution. Finally, signalling molecules usually are present at low to moderate concentration, requiring

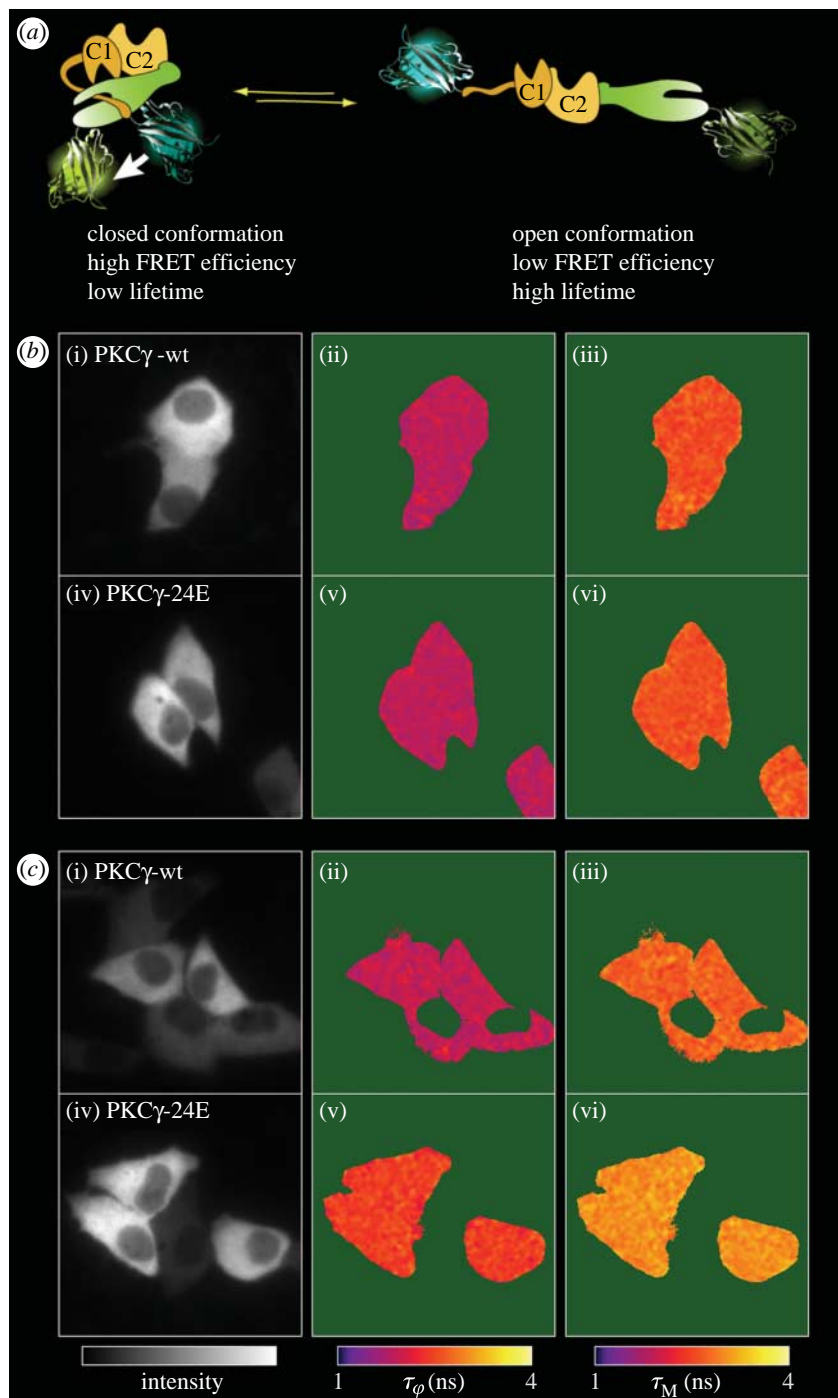


Figure 3. Fluorescence lifetime imaging microscopy reveals differences in PKC conformation. PKC γ was sandwiched between CFP and YFP, and the FRET efficiency was used to distinguish between the open and the closed conformation, as schematically depicted in (a). FLIM experiments (b,c) yield fluorescence intensity images (b(i),(iv),c(i), (iv)), a phase lifetime map (b(ii),(v), c(ii),(v)) and a modulation lifetime map (b(iii),(vi), c(iii),(vi)). Intensity and lifetimes are represented in false-colour according to the scales shown. (b) The wild-type PKC (i–iii) was compared with a mutant that is unable to bind the pseudosubstrate (PKC γ -A24E) (iv–vi), thereby abrogating the closed conformation. However, when ECFP was used as a donor, no difference in donor lifetime was observed between wild-type PKC γ and the mutant A24E, indicating similar FRET efficiencies. (c) On the other hand, when SCFP3A was used as a donor a clear difference between wild-type (i–iii) and the A24E mutant (iv–vi) was observed. The mutant displays a higher fluorescence lifetime and hence a reduced FRET efficiency that fits with an open conformation. Detailed methods and statistical analysis are described elsewhere (Verbeek *et al.* 2008).

sensitive detection. Fluorescence emission can be detected nowadays with the ultimate sensitivity down to the single molecule level (Hell 2007; Walter *et al.* 2008). Hence, it is clear that fluorescence microscopy meets these requirements.

3. PROTEIN KINASE C LOCALIZATION

To study a protein with fluorescence microscopy in cells, it needs to be labelled with a fluorophore. The first *in situ* imaging approaches used purified proteins,

modified with a small organic fluorophore. In the case of intracellular proteins, these molecules need to be reintroduced into the cell, usually by microinjection. This approach was taken to study the relocation and integrity of a classical PKC in living cells (Bastiaens & Jovin 1996).

The cloning of green fluorescent protein (GFP) has revolutionized cell biology, since it is a genetically encoded fluorophore, maturing without the need for a cofactor (Tsien 1998). Evidence from biochemical studies and immunolabelling indicated that PKC translocates from the cytoplasm to the plasma membrane. In a hallmark study, using a GFP-tagged PKC, Meyer and colleagues demonstrated that PKC integrates both calcium and DAG signals (Oancea & Meyer 1998). It was shown that C1 and C2 domains from PKC are individual lipid-binding domains, and that the C1 domain binds DAG at the plasma membrane (Oancea *et al.* 1998) and the C2 domain binds the plasma membrane when calcium levels are increased (Teruel & Meyer 2002). Interestingly, these minimal binding domains fused to GFP can be used as translocation sensors to study lipid turnover in single cells (Schultz *et al.* 2005). Subsequently, numerous sensors based on the same strategy have been reported with varying degrees of specificity and affinity.

Translocation studies require clear separation of the cytoplasmic and membrane fluorescence. By acquiring an optical section by means of confocal microscopy, the out-of-focus contributions of the basal and apical membranes are rejected, thus providing a clear contrast between cytoplasmic and plasma membrane-located fluorescence (figure 2). Increased axial resolution is provided by total internal reflection fluorescence microscopy, which is also known as evanescent wave microscopy (Axelrod 2001; Toomre & Manstein 2001). This type of excitation illuminates a thin layer just above the coverslip (the excitation light decays exponentially with the distance from the coverslip and has a typical thickness of approx. 100 nm) and is very sensitive to fluorescence at the basal membrane. The translocation process is now simply monitored as changed fluorescence intensity. Owing to the excellent axial resolution, this method is very sensitive to small translocation events. Moreover, it can also reveal local translocation events (Codazzi *et al.* 2001), i.e. translocation to specific areas of the plasma membrane.

To investigate the underlying molecular mechanism of spinocerebellar ataxia type 14, a neurodegenerative disease (Yabe *et al.* 2003) which is caused by a mutation in the C1 domain of PKC γ , we performed dual colour imaging experiments using wild-type and mutant PKC. The power of dual colour imaging experiments is that the comparison can be directly made *in situ*, since the data are gathered under identical circumstances, rather than comparing different experiments from different cells (Schultz *et al.* 2005).

The PKCs were fused to either GFP or monomeric red fluorescent protein (mRFP) and co-expressed in cells. The effect of phorbol ester was measured by simultaneously imaging both proteins by confocal microscopy. Subsequently, the translocation of both proteins was quantitatively analysed, and the translocation rates

were compared. These studies revealed that the mutant PKCs have a substantially higher affinity for phorbol ester, whereas the individual (mutated) domains have less membrane affinity (Verbeek *et al.* 2008). This approach was also used to directly compare the translocation rates of binding domains versus full-length proteins (Raghunath *et al.* 2003).

4. PKC CONFORMATION SENSING WITH FRET

Changes in protein activity or interactions are often accompanied by conformation changes. Therefore, it is important to monitor these changes in living cells. To achieve this, the phenomenon of FRET can be used. Principles and applications of FRET are reviewed extensively in the literature (Stryer 1978; Clegg 1996; Gadella *et al.* 1999; Jares-Erijman & Jovin 2003, 2006), and will be only briefly explained here.

FRET is the process in which an excited (donor) fluorophore relaxes back to the groundstate by transferring its energy radiationless to another (acceptor) chromo- or fluorophore. In the case of FRET, the donor fluorescence decreases, the donor excited state lifetime decreases and, if the acceptor is fluorescent, the acceptor fluorescence intensity increases. The most popular methods to measure FRET are based on these observations (Jares-Erijman & Jovin 2003). The best method to measure FRET depends strongly on the application. For example, the FRET efficiency can be accurately quantified by measuring excited state lifetimes, whereas ratio imaging is more suitable for monitoring dynamic FRET changes.

The efficiency of FRET is extremely sensitive to the distance and orientation of the dipole moments of the donor and acceptor fluorophore. For living cell applications, spectral variants of GFP are often used. The most widely used couple is cyan fluorescent protein (CFP)–yellow fluorescent protein (YFP). With the development of monomeric red fluorescent proteins, it has become possible to use red-shifted pairs, e.g. GFP–tag-red fluorescent protein (tagRFP) and YFP–mCherry. Their main advantage is the increased R_0 value that increases the FRET efficiency at a certain distance, or allows the measurement of a larger distance with a similar FRET efficiency (Goedhart *et al.* 2007). The conformation of a protein can be studied by sandwiching the protein of interest between a donor and an acceptor. It may be worthwhile to optimize the efficiency and dynamic range by optimizing the linker length (Hires *et al.* 2008) or the conformation of the fluorescent protein (Nagai *et al.* 2004).

In the case of PKC, the activity of the enzyme is controlled by a pseudosubstrate. The pseudosubstrate occupies the catalytic cleft, and the PKC is in a closed conformation. Once the protein binds its ligands (calcium and DAG or phorbol ester), the pseudosubstrate is released. PKC is in an open conformation and the enzyme can exert its activity (figure 1). To examine whether the closed and open conformation can be identified in single living cells, we compared wild-type PKC with a mutant that is unable to bind its pseudosubstrate (PKC γ -A24E), which therefore represents an open conformation (Pears *et al.* 1990). To measure the conformation of PKC in

living cells by means of FRET, we fused the N-terminus to enhanced cyan fluorescent protein (ECFP) and the enhanced yellow fluorescent protein (EYFP) to the C-terminus, as schematically shown in figure 3a.

To quantify the FRET efficiency in single living cells, we employed frequency domain fluorescence lifetime imaging microscopy (FLIM) that measures the excited state lifetime. This method yields a phase lifetime (τ_ϕ) determined from the phase shift of the emission light and a modulation lifetime (τ_M) determined from the decrease in modulation depth of the emission light relative to the excitation light.

A decrease in the excited state lifetime of the donor indicates FRET and the FRET efficiency can be calculated by comparison with the control lifetime of the donor, i.e. in the unquenched state (Vermeer *et al.* 2004; van Munster & Gadella 2005). A control donor phase and modulation lifetime for ECFP was measured of 2.2 and 2.9 ns, respectively. As can be inferred from figure 3a for both constructs, the phase and modulation lifetime reduced by 0.3 ns, indicating that FRET occurs. However, no difference in FRET between the wild-type and mutated PKC was observed (figure 3b). We hypothesized that the tendency of ECFP and EYFP to interact (Zacharias *et al.* 2002) obscured the changed conformation. Therefore the ECFP in both constructs was exchanged for SCFP3A, an optimized CFP variant with the A206K mutation that abolishes the interaction with YFP (Zacharias *et al.* 2002; Kremers *et al.* 2006). The wild-type PKC still showed substantial FRET, whereas the mutant showed an increased CFP lifetime, corresponding to a decrease in FRET efficiency (figure 3c). These results demonstrate that a difference in PKC conformation can be measured in cells and stress the importance of using monomeric GFPs. Interestingly, mutants that cause ataxia also showed a decreased FRET signal, demonstrating that the mutants have an altered conformation in living cells (Verbeek *et al.* 2008).

We hypothesized that phorbol esters would change the PKC conformation from a closed (high FRET) conformation to an open (low FRET) conformation. Surprisingly, when the effect of phorbol ester was tested, an increase of FRET was observed (data not shown). This observation probably reflects phorbol ester-induced plasma membrane accumulation that concentrates PKC on a two-dimensional surface, causing intermolecular FRET, also known as bystander FRET. This effect is always of concern when FRET is measured between (peripheral) membrane proteins, but it is possible to differentiate between bystander FRET and specific FRET between two membrane-located proteins by competition with proteins that are not tagged with a FRET acceptor (Adjobo-Hermans *et al.* 2006).

5. PKC ACTIVITY MEASURED BY FRET

The actual enzymatic activity of a (signalling) enzyme is in many cases the most interesting parameter. The activity transduces the signal to downstream effectors. Enzymatic activities can be transient and local and, hence, microscopy methods are required to determine the spatio-temporal characteristics of the activity. An elegant method to detect activated PKC

was published by Ng *et al.* (1999) using GFP-PKC α and a fluorophore-tagged antibody directed against phosphorylated PKC as a FRET acceptor. The presence of FRET detected by FLIM was used to quantitatively measure the phosphorylation status of PKC α that in turn correlates with the activation of the enzyme. However, to perform these experiments in living cells, the antibody needed to be micro-injected into cells. Less invasive and less labour-intensive approaches have since been developed. The most popular and widely used FRET-based probes detecting activity are completely genetically encoded. This allows transfection of plasmids into cells and subsequent analysis in living cells, tissue or whole animals (Heim *et al.* 2007).

The first straightforward application of a genetically encoded FRET-based biosensor for enzymatic activity was to detect cleavage of a fusion protein (Heim & Tsien 1996; Mitra *et al.* 1996). This approach has been widely adopted to measure the activity of proteases that are activated during apoptosis (Rehm *et al.* 2002; Takemoto *et al.* 2003). More sophisticated approaches are usually necessary to develop sensors for kinase activity, nucleotide exchange activity or small molecules, such as calcium (Miyawaki *et al.* 1997; Romoser *et al.* 1997), cAMP (Ponsioen *et al.* 2004) and DAG (Violin *et al.* 2003; Sato *et al.* 2006).

A typical sensor consists of a sensor part that is sandwiched between a donor and an acceptor fluorophore. The conformation change of the sensor is translated into a change in FRET that can be measured in single cells. Although, the FRET efficiency of a sensor can be quantified, it is predominantly used to detect a change in FRET efficiency. The most employed method to monitor FRET changes in single living cells is ratio imaging. This method is based on measuring changes in donor and sensitized acceptor fluorescence intensity due to changes in FRET. FRET changes can be measured with high temporal resolution (down to ms).

Genetically encoded sensors have several interesting features: (i) their subcellular location can be altered (Llopis *et al.* 1998), (ii) their sensitivity can be tuned by introducing mutations (Miyawaki *et al.* 1997), and (iii) their dynamic range can be optimized. Since FRET is an orientation-sensitive process, the FRET response can be optimized by altering the relative orientation of the dipole moment of the donor and acceptor probe. This can be achieved by using circularly permuted fluorescent proteins (Nagai *et al.* 2004). Another approach is to systematically alter the length of the linker between the probes and the sensor region (Hires *et al.* 2008). Finally, the FRET efficiency generally increases when red-shifted probes are used (Goedhart *et al.* 2007). The effects of changing orientation, linkers or probes are generally unpredictable. Therefore, it is essential to be able to quickly construct and analyse several constructs during the optimization process.

Two types of FRET-based sensors for measuring PKC activity are available. KCP is based on pleckstrin, a PKC substrate, sandwiched between two GFP variants. It is reported to be sensitive for novel PKC types. Phosphorylation of the pleckstrin induces

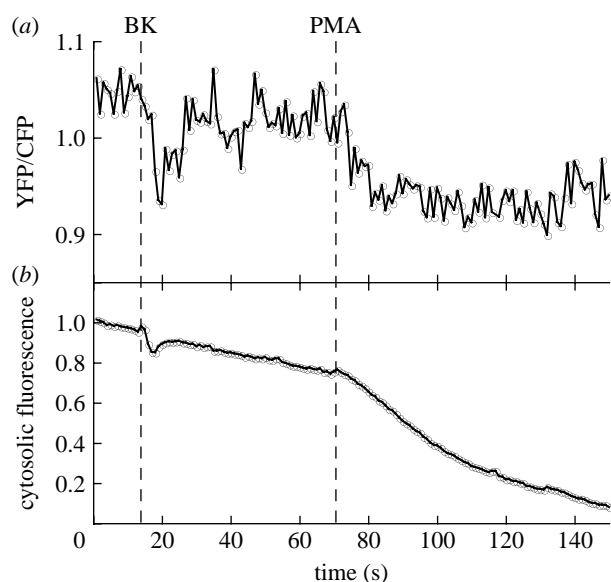


Figure 4. Parallel measuring of PKC translocation and activity in N1E-115 cells. (a) The activity of PKC was monitored using the FRET probe CKAR and (b) the translocation using an RFP-tagged PKC β . Cells were stimulated with 1 μ M bradykinin (indicated by BK) and subsequently with 100 nM phorbol ester (indicated with PMA). The steady decrease observed in the translocation measurement is due to photobleaching. Experiments were performed on a Leica SP2 confocal microscope in sequential scanning mode to prevent fluorescence bleedthrough.

conformational changes that can be monitored by FRET (Schleifenbaum *et al.* 2004). The other probe, C kinase activity reporter (CKAR), is based on a substrate sequence optimal for PKC and a phosphopeptide-binding domain that binds the phosphorylated substrate, causing a loss in FRET (Violin *et al.* 2003). Both probes can readily be used to monitor dynamics of PKC activity in living cells. Interestingly, it was demonstrated that a membrane-targeted version of CKAR, pmCKAR, was capable of detecting oscillatory phosphorylation by PKC (Violin *et al.* 2003). In a follow-up study, the CKAR was targeted to a variety of cellular compartments, to analyse the phosphorylation kinetics in each of the locations, showing that these kinetics are rather different (Gallegos *et al.* 2006). These studies demonstrate that, besides the spatial information attained by microscopy, the targeting of a genetically encoded sensor to a specific subcellular location is a powerful approach to extract information from specific regions of interest within the cell.

Figure 4 shows the results of an experiment in which the translocation of PKC is measured simultaneously with its activity. The activity was measured using the FRET probe CKAR, whereas an RFP-tagged PKC was used to monitor translocation. Several interesting observations are made. The translocation dynamics closely follow the PKC activity, and the bradykinin-induced translocation/activation is very transient. Hence, the activity of PKC is not persistent, probably due to the high activity of phosphatases. The translocation amplitude induced by bradykinin is low, relative to the effect of phorbol ester that induces full transloca-

tion. On the other hand, the activity of PKC induced by bradykinin is similar to that induced by phorbol ester. These observations indicate that the FRET sensor provides a maximal response when the translocation of PKC is submaximal. Therefore, the FRET sensor is rather sensitive to PKC activity. Whether natural substrates of PKC have a similar sensitivity remains to be shown.

It is of note that other methods of measuring PKC activation exist; for instance, when the substrate changes location, which is the case for the PKC substrate myristoylated alanine-rich C kinase substrate (MARCKS) (Sawano *et al.* 2002). This protein translocates from the membrane to the cytoplasm upon phosphorylation that can be followed in living cells. Also more downstream effectors, such as mitogen-activated protein (MAP) kinases, can be used as indicator of PKC activity (Verbeek *et al.* 2008).

6. FUTURE

The above-described approaches and methodologies have yielded a wealth of new information on the process of signal transduction in single living cells. Technological advances will be instrumental for creating new methods and improving current methods. For example, it is important to increase spatial and temporal resolution and sensitivity of microscopes, while minimizing phototoxicity (Hoebe *et al.* 2007), to reveal details on dynamic organization of signalling complexes (Hell 2007; Walter *et al.* 2008). Probe development is another very important and active area. It aims at improving brightness and folding of fluorescent proteins and creation of probes with specific characteristics (e.g. photoactivatable and photoswitchable proteins). It also includes application of quantum dots and synthesis of small organic probes that can be used to tag specific small genetically encoded tags (Giepmans *et al.* 2006). In general, the development of a sensitive FRET sensor that minimally disrupts cellular physiology can be a difficult task. It usually requires the construction, screening and characterization of multiple constructs. Therefore, automation of several steps in this procedure can speed up sensor development. In addition, the development and improvement of blue- and red-shifted FRET pairs is essential and will allow the detection of multiple FRET probes in a single cell, which will enable the output of the two probes to be correlated in time and space (Ai *et al.* 2008; Piljic & Schultz 2008). We anticipate that these types of experiments will become as widely used as ordinary multicolour imaging. Ultimately, by combining multiple FRET probes, it will be possible to measure translocation, activation and conformation in a single cell with high sensitivity and temporal resolution. The quantitative data provided by cellular imaging studies can be used as input for mathematical models, describing a signalling pathway (Megason & Fraser 2007; Cheong & Levchenko 2008). These models will be useful to comprehend the signalling cascade. Moreover, the models will be instrumental for the design of new experiments.

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