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*Published in:*  
Biochemical and Biophysical Research Communications

*DOI:*  
[10.1016/j.bbrc.2007.09.123](https://doi.org/10.1016/j.bbrc.2007.09.123)

2007

[Link to publication](#)

*Citation for published version (APA):*  
Kazi, J. U., & Soh, J. W. (2007). Isoform-specific translocation of PKC isoforms in NIH3T3 cells by TPA. *Biochemical and Biophysical Research Communications*, 364(2), 231-237.  
<https://doi.org/10.1016/j.bbrc.2007.09.123>

*Total number of authors:*  
2

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**Citation information:** Kazi J. U. and Soh J. W. (2007) Isoform-specific translocation of PKC isoforms in NIH3T3 cells by TPA. *Biochemical and Biophysical Research Communications* 364(2), 231-237

**Link to the original article:** <http://dx.doi.org/10.1016/j.bbrc.2007.09.123>

## Isoform-specific translocation of PKC isoforms in NIH3T3 cells by TPA

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### Abstract

Protein kinase C (PKC), a multi-gene family of enzymes, plays key roles in the pathways of signal transduction, growth control and tumorigenesis. Variations in the intracellular localization of the individual isoforms are thought to be an important mechanism for the isoform-specific regulation of enzyme activity and substrate specificity. To provide a dynamic method of analyzing the localization of the specific isoforms of PKC in living cells, we generated fluorescent fusion proteins of the various PKC isoforms by using the green fluorescent protein (GFP) as a fluorescent marker at the carboxyl termini of these enzymes. The intracellular localization of the specific PKC isoforms was then examined by fluorescence microscopy after transient transfection of the respective PKC–GFP expression vector into NIH3T3 cells and subsequent TPA stimulation. We found that the specific isoforms of PKC display distinct localization patterns in untreated NIH3T3 cells. For example, PKC $\alpha$  is localized mainly in the cytoplasm while PKC $\epsilon$  is localized mainly in the Golgi apparatus. We also observed that PKC $\alpha$ ,  $\beta$ 1,  $\beta$ 2,  $\gamma$ ,  $\delta$ ,  $\epsilon$ , and  $\eta$  translocate to the plasma membrane within 10 min of the start of TPA treatment, while the cellular localizations of PKC $\zeta$  and  $\iota$  were not affected by TPA. Using a protein kinase inhibitor, we also showed that the kinase activity was not important for the translocation of PKC. These results suggest that specific PKC isoforms exert spatially distinct biological effects by virtue of their directed translocation to different intracellular sites.

### Keywords:

PKC; Translocation; Localization; TPA; NIH3T3

### Introduction

Protein kinase C (PKC) is a multi-gene family that encodes 10 distinct isoforms of lipid-regulated serine/threonine kinases [1]. PKC isoforms play pivotal roles in several signal transduction pathways that regulate cellular growth, transformation, differentiation and apoptosis. PKC can be activated by calcium or by various phospholipids, diacylglycerol (DAG), generated by phospholipase C or phospholipase D, and by fatty acids, generated by phospholipase A2, depending on the PKC isoforms [2,3].

PKC isoforms can be classified into three groups, based on their structure and cofactor requirement: classical PKC isoforms ( $\alpha$ ,  $\beta$ 1,  $\beta$ 2,  $\gamma$ ) which are regulated by DAG and Ca<sup>2+</sup>, novel PKC isoforms ( $\delta$ ,  $\epsilon$ ,  $\eta$ ,  $\theta$ ) which are regulated by DAG, but not by Ca<sup>2+</sup>, and atypical PKC isoforms ( $\zeta$ ,  $\iota$ ) which are not responsive to either DAG or Ca<sup>2+</sup>. Each of these isoforms consists of an amino-terminal regulatory domain and a carboxy-terminal catalytic domain. Classical and novel PKC isoforms contain a tandem repeat of cysteine-rich motif referred to as the C1a and C1b domain. The C1a and C1b domains are the binding sites for DAG and the C1b domain is responsible for the majority of high affinity DAG binding, whereas atypical PKC isoforms, which lack the C1b domain, cannot bind DAG. Classical PKC isoforms are also characterized by a C2 domain that binds anionic phospholipids in a calcium-dependent way. The C2 domain is also present in novel PKC isoforms, but because of its lack of calcium binding residues, the novel C2 domain cannot bind either calcium or phospholipids [1]. The C2 domain of the novel

PKC isoform, PKC $\delta$ , has been shown to be a phosphotyrosine binding domain [4] and the question as to whether other C2 domains are also phosphotyrosine binding domains, is still being investigated.

PKC isoforms show different patterns of subcellular localization that can differ for the different isoforms according to the tissue and cell type. Moreover, PKC isoforms change their subcellular localization upon activation and their biological activities depend on their subcellular localization. However, it is not clear how the isoform-specific subcellular localization and stimulus-induced translocation can be achieved. Probably, PKC binding proteins play important roles in this process and the binding of DAG to C1 domains is also important for the localization of PKC isoforms [2]. Several phorbol esters are analogous to DAG and can bind and activate all PKC isoforms, except the atypical PKC isoforms.

Several studies have demonstrated that phorbol esters such as TPA (12-O-tetradecanoyl-phorbol-13-acetate) can translocate specific PKC isoforms to a specific cellular compartment. PKC $\gamma$ , a classical PKC isoform, was reported to be translocated from the cytosol to the plasma membrane in COS-7 cells by TPA [5], whereas PKC $\alpha$  was redistributed in rat liver WB cells from the cytosol to the plasma membrane and nucleus by the same stimuli [6], although classical PKC isoforms are not known to contain any of the known nuclear localization signals. The macro-cyclic lactone, bryostatin 1, activate PKC by binding with the C1 domain, was also reported to translocate PKC $\alpha$  from the cytosol to the plasma membrane [7].

PKC $\delta$ , a novel PKC isoform, was shown to be localized to the cytoplasm and nucleus in unstimulated CHO-K1 cells and redistributed to the plasma membrane and nuclear membrane after TPA stimulation [8], whereas, in HCT116 cells, PKC $\delta$  was redistributed from the cytosol to the plasma membrane [9]. PKC $\epsilon$  exhibits a unique association with Golgi membranes via its C1 domain and modulates Golgi functions [10]. Another novel PKC isoform, PKC $\gamma$ , was reported to localize at the Golgi apparatus, the endoplasmic reticulum and the nuclear envelope in COS-7 and MCF-7 cells, and TPA induced its translocation to the plasma membrane and the nuclear envelope [11]. These studies suggest that the subcellular localization of PKC isoforms is not only isoform or cell line specific, but also stimuli specific.

Several techniques have been developed to study the subcellular localization of signaling proteins. Green fluorescent protein (GFP) fusion proteins facilitate the process of monitoring the protein's translocation or subcellular localization. The translocation behaviors of GFP-fusion proteins are similar to those of the native proteins. Herein, we report the direct visualization of the localization of individual PKC isoforms in living NIH3T3 cells. We study the translocation of individual PKC isoforms by TPA stimulation. Since their stimulation with TPA for a long time causes the down-regulation of the PKC isoforms through proteolytic degradation, we used a short time activation method. We also demonstrated the role of the PKC inhibitor in TPA induced PKC localization.

## Materials and methods

**Plasmids:** pGFP3 was used to generate PKC mutants with an N-terminal GFP fusion protein. pGFP3 is a mammalian expression vector that contains a CMV promoter, Kozak translation initiation sequence, start codon, EcoR1 and BamH1 cloning sites, C-terminal GEP cDNA sequence and stop codon. It was generated by ligating the annealed synthetic oligonucleotides (upper strand, 5'-AGCTTGCCACCATGGAATTCCG GATCC-3'; lower strand, 5'-GATCGGATCCGAATTCCATGGT GGCA-3') into pEGFP-N1 (BD Biosciences) after

digestion with HindIII and BamHI. pGFP3-PKC $\alpha$ -WT, pGFP3-PKC $\gamma$ -WT, pGFP3-PKC $\delta$ -WT, pGFP3-PKC $\varepsilon$ -WT, pGFP3-PKC $\eta$ -WT, pGFP3-PKC $\zeta$ -WT, and pGFP3-PKC $\iota$ -WT were constructed by sub-cloning EcoRI fragments of the full length open reading frames of the respective PKC isoforms into pGFP3 expression vector. pGFP3-PKC $\beta$ 1-WT and pGFP3-PKC $\beta$ 2-WT were constructed by sub-cloning the BamHI fragments of the full length open reading frames of the PKC $\beta$ 1 and PKC $\beta$ 2 isoforms into pGFP3 expression vector.

**Cell Transfection:** NIH3T3 cells were grown in DMEM containing 10% calf serum. Triplicates of  $1 \times 10^5$  cells in 35 mm plates were transfected by lipofectin (Invitrogen) with 5  $\mu$ g of the expression plasmid. pGFP3 was used as an empty control vector. COS-7 cells were grown in DMEM containing 10% fetal bovine serum. About  $1 \times 10^5$  cells in 60 mm plates were transfected by lipofectin with 5  $\mu$ g of the expression vectors or control vector.

**Fluorescence microscopy:** Twenty-four hours after transfection in serum-free medium, Hoechst 33258 was added to the medium at a concentration of 1  $\mu$ g/ml and allowed to stand for 30 min to stain the nuclei. The plates were then placed under a fluorescent microscope (Inovision Digital Deconvolution System) for real time observation. TPA was added to the medium at a concentration of 100 ng/ml to stimulate PKC translocation. Immediately following the TPA treatment, fluorescent images from both the green (GFP) and blue (nuclei) were recorded by the automated Inovision System. PKC-GFP was imaged by excitement with 488 nm light and the emitted light was measured at 509 nm.

**Western blotting:** Six hours after transfection, COS7 cells were fed with DMEM containing 10% calf serum and left to stand overnight. The cells were then transferred to 10 cm plates and grown for 24h before protein extraction. The cellular proteins were extracted by cell lysis in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate, 0.5% deoxycholate, 2mM EDTA, 2mM EGTA, 1mM dithiothreitol) containing protease inhibitors (10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, 0.1 mM PMSF) and phosphatase inhibitors (1 mM NaF, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM  $\beta$ -glycerophosphate). The GFP-fusion proteins were immunoprecipitated from 300  $\mu$ g of the cell extracts using 3  $\mu$ g of anti-GFP antibody and 30  $\mu$ l of protein G-Sepharose, after 3h of incubation at 4DC and analyzed by SDS-PAGE and an Enhanced Chemiluminescence Western Blotting System (Amersham).

## Results

### Generation of C-terminal GFP fusion PKC isoforms:

Green fluorescent proteins are extensively used to monitor translocation or subcellular localization of proteins in living cells. To observe the subcellular localization of PKC, we constructed the C-terminal GFP fusion PKC isoforms. The expression plasmids, pGFP3-PKC $\alpha$ -WT, pGFP3-PKC $\beta$ 1-WT, pGFP3-PKC $\beta$ 2-WT, pGFP3-PKC $\gamma$ -WT, pGFP3-PKC $\delta$ -WT, pGFP3-PKC $\varepsilon$ -WT, pGFP3-PKC $\eta$ -WT, pGFP3-PKC $\zeta$ -WT, and pGFP3-PKC $\iota$ -WT, were generated by sub-cloning PKC $\alpha$ ,  $\beta$ 1,  $\beta$ 2,  $\gamma$ ,  $\delta$ ,  $\varepsilon$ ,  $\eta$ ,  $\zeta$  and  $\iota$  cDNAs, respectively, into the vector pGFP3 (for details see Material and methods). The pGFP3-PKC-WT plasmids encode the C-terminal green fluorescent protein fused to full length open reading frames of the PKC isoforms (Supplementary figure 1A). COS-7 cells were transfected with the control vector, pGFP3, or expression plasmids. The expression levels of the overexpressed PKC-GFP proteins were examined by western blotting using anti-GFP antibody. The plasmids expressed the corresponding GFP fusion proteins with the expected sizes (Supplementary figure 1B). Then, GFP was expressed alone in NIH3T3 cells using the control vector, pGFP3, and its localization was observed with fluorescence microscopy. GFP was expressed in the cytoplasm and nucleus

and after TPA stimulation no translocation was observed (Supplementary figure 1C). The translocation patterns described below for PKC–GFP therefore appear to reflect the intact fusion protein.

### **Real time analysis of PKC $\beta$ translocation in NIH3T3 cells:**

Previous studies have suggested that the stimulation of various PKC isoforms by PKC agonist phorbol esters results in the rapid translocation of the isoforms from the cytosolic sites to membrane-associated sites [11–13]. To study the real time translocation of PKC isoform, we used the classical PKC isoform, PKC $\beta$ , which has two transcriptional variants (PKC $\beta$ 1, PKC $\beta$ 2) containing about 96% homology. NIH3T3 cells were transfected with pGFP3PKC $\beta$ 1-WT and pGFP3-PKC $\beta$ 2-WT, which encoded PKC $\beta$ 1-GFP and PKC $\beta$ 2-GFP, respectively. Several investigators demonstrated that GFP-fusion PKC isoforms show similar levels of activation and a similar extent of translocation to that of PKC alone [5,14,15]. The cells were placed under the fluorescent microscope and stimulated by 100 ng/ml TPA. Images were taken every 20 s for 20 min. Before their stimulation, PKC $\beta$ 1-GFP (Fig.1 0min) and PKC $\beta$ 2-GFP (Fig. 2 0min) were diffusely distributed in the cytosol suggesting that these isoforms are present in abundant amounts in the cytosol of the unstimulated cells, in fact designating inactive enzymes. The TPA-induced activation of the PKC isoforms resulted in their translocation to the cell membrane. The complete translocation of PKC $\beta$ 1-GFP from the cytosol to the plasma membrane was observed after 12min (Fig. 1; Supplementary movie 1). In contrast, the TPA treatment of PKC $\beta$ 2-GFP over-expressing cells results in the apparently faster translocation of PKC $\beta$ 2-GFP to the membrane fraction (Fig. 2; Supplementary movie 2). These results suggest that the PKC $\beta$  isoforms differ with respect to their responsiveness to activation by TPA, even though their regulatory domains are the same. After TPA treatment, the shape of the cells overexpressing PKC $\beta$  dramatically changed, moreover the PKC $\beta$ 2 overexpressing cells displayed unusual membrane blebbing after 12min of stimulation.

### **TPA induced translocation of PKC isoforms in NIH3T3 cells:**

It is now widely accepted that the biological functions of PKC isoforms depend on their intracellular localization. PKC isoforms can be localized to multiple cellular compartments, including the plasma membrane, endosomes, Golgi, nucleus and nuclear membrane. To study the sub-cellular localization of other PKC isoforms, we transfected the pGFP3-PKC $\alpha$ -WT, pGFP3-PKC $\gamma$ -WT, pGFP3PKC $\delta$ -WT, pGFP3-PKC $\epsilon$ -WT, pGFP3-PKC $\eta$ -WT, pGFP3-PKC $\zeta$ -WT, pGFP3-PKC $\iota$ -WT plasmids into NIH3T3 cells. After transient expression, the subcellular localization of the GFP fusion proteins was observed with fluorescence microscopy. With a few exceptions, the unstimulated cells showed the diffuse distribution of each PKC isoform throughout the cytoplasm (Fig.3), but after TPA stimulation, each enzyme displayed a unique relocalization pattern. After 10 min of treatment with 100 ng/ml TPA, the activated PKC $\alpha$  mostly localized to the plasma membrane. A small portion of the activated PKC $\alpha$  was concentrated near the nucleus, a pattern like endoplasmic reticulum (Fig. 3B), suggesting that PKC $\alpha$  is associated with the endoplasmic reticulum (ER) membrane proteins. PKC $\alpha$  did not appear to localize to either the nuclear membrane or nucleus. The other classical PKC isoform, PKC $\gamma$ , was distributed throughout the cytoplasm of the untreated cells, showing a distinct association with the Golgi like structures, without any nuclear accumulation (Fig. 3C), suggesting that inactive PKC $\gamma$  expressed in the cytoplasm can accumulate in the Golgi-like structures. Upon TPA stimulation, PKC $\gamma$  translocated to the plasma membrane with cis-Golgi redistribution (Fig.3D).

PKC $\delta$ , a novel PKC isoform, was localized throughout the cytoplasm and also concentrated in perinuclear regions such as the Golgi in the unstimulated cells (Fig.3E). Upon TPA treatment, the cells noticeably and rapidly became rounded with considerable shrinking, and the cytoplasmic proteins translocated to the plasma and nuclear membrane with Golgi redistribution (Fig.3F). The other novel PKC isoform, PKC $\epsilon$ , was expressed in the cytosol with a diffused distribution throughout the cytoplasm(Fig.3G) and, after TPA treatment, translocated to the plasma membrane with significant localization to the Golgi like structures (Fig. 3H). PKC $\eta$  existed mainly in the Golgi apparatus with granular cytoplasmic distribution in the unstimulated cells (Fig.3I). PKC $\eta$  expressed in COS7, was previously reported to be localized in the Golgi apparatus [11], further supporting our observation. TPA treatment induced the translocation of PKC $\eta$  to the plasma membrane with cytoplasmic distribution (Fig. 3J), suggesting that activated PKC $\eta$  might be localized to cytoplasmic membrane like structures. The atypical PKC isoform, PKC $\zeta$ , was found to be diffusely distributed in the cytosol (Fig.3K). In contrast, PKC $\iota$  was strongly expressed in the nucleus with cytoplasmic diffusion (Fig. 3M). Upon TPA treatment, the atypical PKC isoforms did not translocate significantly (Fig. 3L and N).

#### **Effect of staurosporine on TPA induced translocation:**

In order to explore the role of the kinase activity of PKC isoforms in TPA-mediated translocation, we used staurosporine, a potent inhibitor of PKC isoforms. Staurosporine binds with the kinase domain of PKC that interferes in the binding of ATP, resulting in the inhibition of kinase activity [16,17]. NIH3T3 cells were transfected with the pGFP3PKC $\alpha$ -WT, pGFP3-PKC $\beta$ 1-WT, pGFP3-PKC $\beta$ 2-WT, pGFP3-PKC $\gamma$ -WT, pGFP3-PKC $\delta$ -WT, pGFP3-PKC $\epsilon$ -WT plasmids. After transient expression, the cells were treated with staurosporine followed by TPA. The classical PKC isoforms were mostly localized to the plasma membrane, demonstrating that kinase activity is not essential for the translocation of the classical PKC isoforms (Fig. 4A–D). PKC $\delta$  and PKC $\epsilon$  were also more abundant in the plasma membrane with a small amount of diffusion into the cytosolic membranes (Fig.4E and F). These results suggest that the translocation of PKC isoforms is regulated by the regulatory domain and that staurosporine increases the plasma membrane translocation.

#### **Discussion**

Several factors, such as the specific isoform, cell type and activating ligand, which account for the diversity of biological effects, form the central focus in the PKC field. The differences in the substrate preferences of PKC isoforms may contribute to this diversity. Another mechanism of localization may be the involvement of the interacting proteins. Using GFP-fusion PKC isoforms, several studies have shown the existence of a unique pattern of translocation of PKC isoforms in living cells in response to different physiological activators. Sakai et al [5] described the translocation patterns of PKC $\gamma$  in response to several stimuli using GFP-fusion PKC $\gamma$ . Distinctive patterns of translocation of GFP-fusion PKC $\delta$  have also been reported in CHO-K1 cells [8]. In this study, we studied the differential localization of PKC isoforms in the form of green fluorescent protein fusion proteins in NIH3T3 cells by over-expressing each isoform. Our results (summarized in Supplementary table 1) suggest that, upon their activation by TPA, the classical and novel PKC isoforms translocate to the plasma membrane with a distinct cytosolic membranous co-localization. All of the isoforms are present in abundant amounts in the cytosol in the untreated cells, presumably indicating they are inactive PKC proteins.

Cytosolic membranes such as the Golgi and ER appear to be a target for the localization of

several PKC isoforms. The co-localization of PKC $\alpha$  to the ER with plasma membrane is in agreement with the results of previous studies in 3T3 cells [18,19]. Although some previous studies reported the TPA-induced translocation of PKC $\alpha$  to the nuclear boundary [20] or nucleus [12], we did not observe any nuclear localization of PKC $\alpha$ . One possible reason for these findings might be the difference in the cell line or culture condition. The localization of PKC $\gamma$  and PKC $\delta$  differs from that of the other isoforms in that these two isoforms localize to the cytoplasmic subcellular organelles in unstimulated cells, indicating the activation of a portion of these isoforms. The rapid morphological changes of PKC $\beta$  and PKC $\delta$  overexpressing cells after TPA stimulation might be the reason for the severe retardation of cell proliferation that has also been reported previously [21]. These morphological changes were also observed in the staurosporine treated cells suggesting that the kinase activity of the PKC isoforms is indispensable for cell proliferation.

PKC $\eta$ , a novel PKC, localized to the Golgi and other cytoplasmic membranous organelles and its TPA-induced translocation to the plasma membrane has also been supported by other investigators [11]. The Golgi localization of the inactive PKC isoforms was shown to be mediated by the C1b domain [10,11]. However it is not clear how the C1b domain mediates this localization. Another possible explanation for the Golgi localization of the PKC isoforms might be the association of the close PKC homolog, PKD, which was shown to interact with the novel PKC isoforms through its PH-domain [22,23] and existing in the Golgi, regulating Golgi-related functions [24].

The results of our studies using the kinase inhibitor, staurosporine, suggesting that the regulatory domain of the PKC isoforms, but not the full-length enzyme, is obligatory for PKC localization, are also supported by the findings of Maissel et al., who found that the kinase-dead mutant of PKC $\eta$  showed similar localization to that of the wild-type [11]. The results presented herein demonstrate that the localization of PKC isoforms varies from isoform to isoform and, thus, the PKC family may not to be a functionally redundant protein kinase, but rather each isoform may have a distinct physiological role that can be visualized by the GFP fusion protein.

### **Acknowledgment**

This work was supported by Korea Research Foundation grant (KRF-2004-003-C00128).

### **Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version.

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### **Figure legends:**

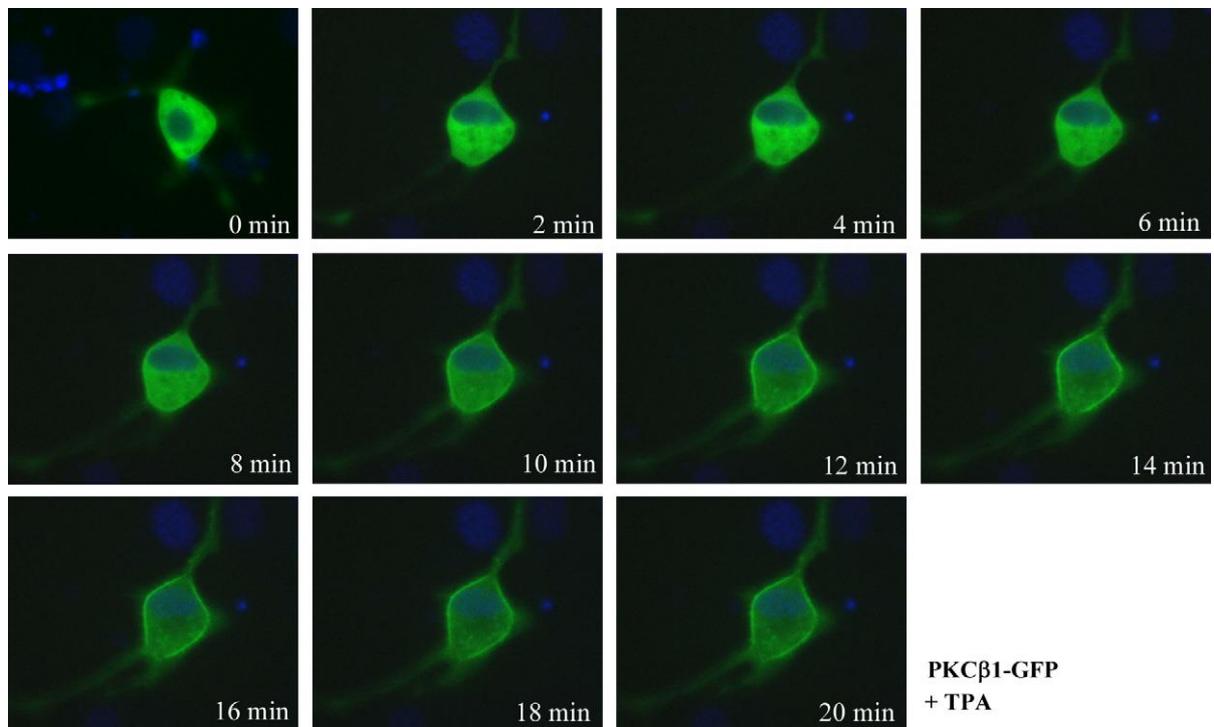
**Figure 1:** Real time analysis of PKC $\beta$ 1 translocation. NIH3T3 cells were transfected with pGFP3-PKC $\beta$ 1-WT plasmid. After 24h of transfection in serum free medium, the nucleus was stained with Hoechst 33258 for 30 min and treated with 100 ng/ml TPA. Fluorescent images of NIH3T3 cells expressing PKC $\beta$ 1-GFP with TPA stimulation were taken.

**Figure 2:** Real time analysis of PKC $\beta$ 2 translocation. NIH3T3 cells were transfected with pGFP3-PKC $\beta$ 2-WT plasmid. After 24h of transfection in serum free medium, the nucleus was stained with Hoechst 33258 for 30 min and treated with 100 ng/ml TPA. Fluorescent images of NIH3T3 cells expressing PKC $\beta$ 2-GFP with TPA stimulation were taken.

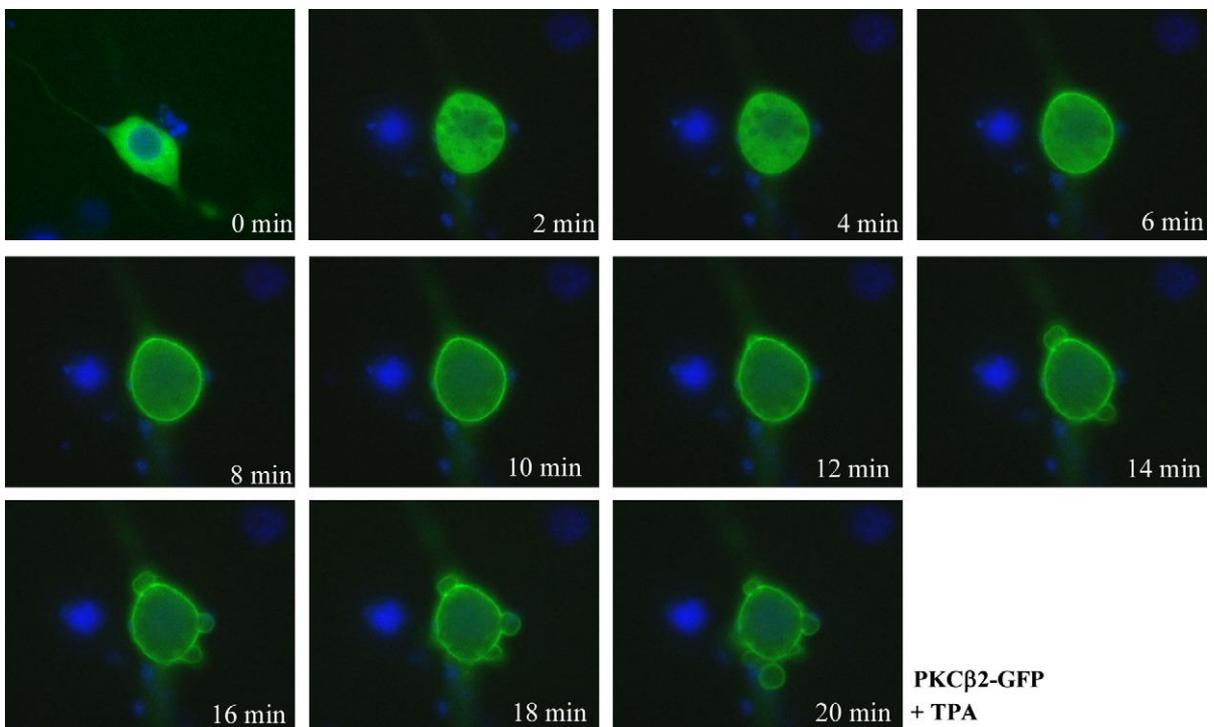
**Figure 3:** TPA induced translocation of PKC isoforms. NIH3T3 cells were transfected with pGFP3-PKC-WT plasmid. After 24h of transfection in serum free medium, the nucleus was stained with Hoechst 33258 for 30 min. Fluorescent images of NIH3T3 cells expressing PKC-GFP before stimulation and after 10 min of TPA stimulation were taken.

**Figure 4:** Effect of staurosporine on TPA induced translocation. NIH3T3 cells were transfected with pGFP3-PKC-WT plasmid or empty control vector. After 24h of transfection in serum free medium, the nucleus was stained with Hoechst 33258 for 30 min, then treated with staurosporine followed by 10 min of TPA stimulation. Fluorescent images of NIH3T3 cells expressing PKC-GFP were taken.

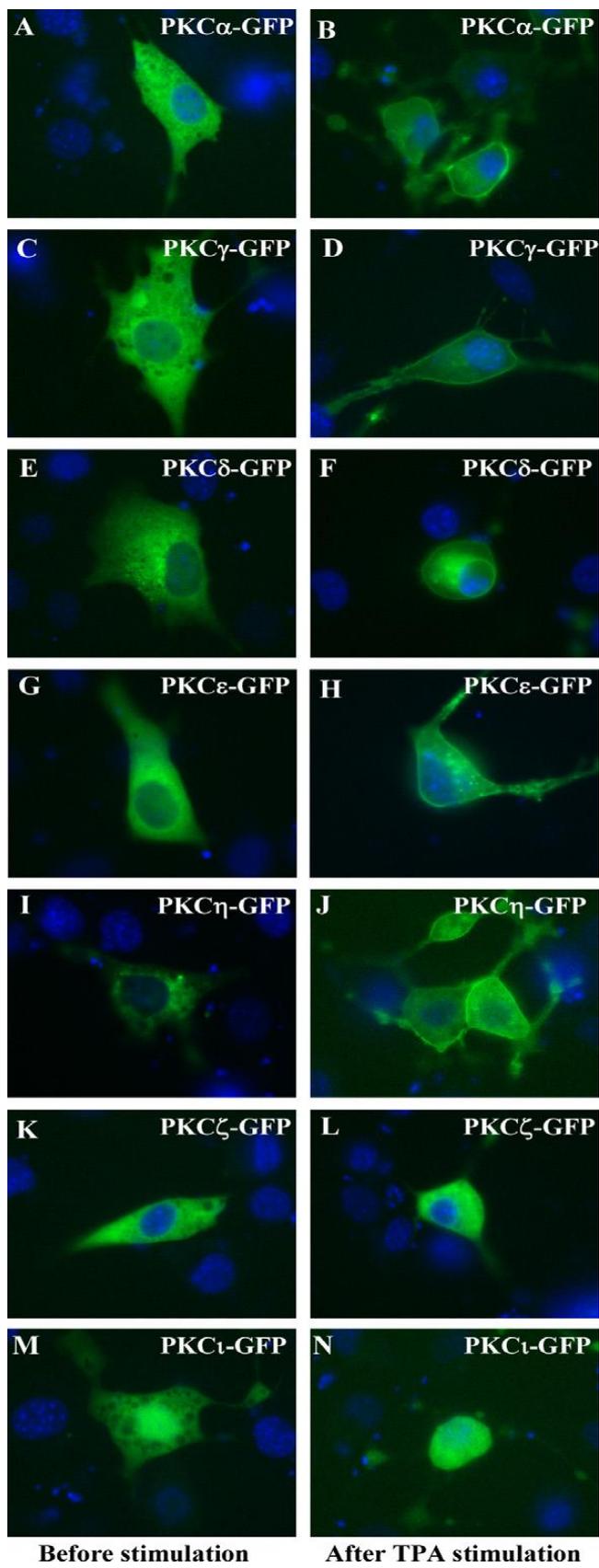
**Figure 1**



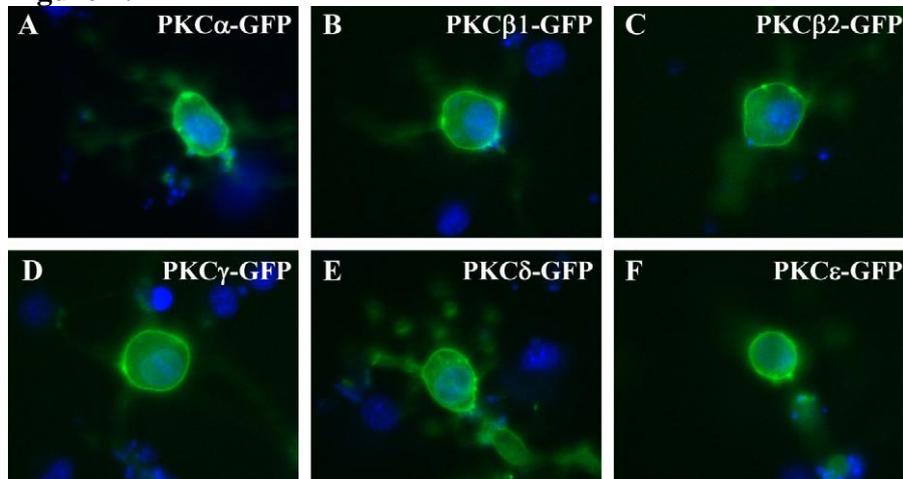
**Figure 2:**



**Figure 3:**



**Figure 4:**



**Supplementary figure legends:**

**Supplementary Figure 1.**

Generation of C-terminal GFP fusion PKC isoforms. Structure of the C-terminal GFP fusion PKC isoforms. Other than the PKC $\beta$ 1 and PKC $\beta$ 2, PKC isoforms were ligated as EcoRI fragments and PKC $\beta$ 1 and PKC $\beta$ 2 were ligated as BamHI fragments. (B) COS-7 cells were transfected with pGFP3-PKC-WT plasmid or empty control vector and cellular proteins were extracted by cell lysis. Western blotting was performed using anti-GFP-antibody. (C) Fluorescent images of NIH3T3 cells expressing GFP before stimulation and after 10 min of TPA stimulation. NIH3T3 cells were transfected with pGFP3 empty control vector. After 24 h of transfection in serum free medium nucleus was stained with Hoechst 33258 for 30 min.

Supplementary figure 1:

