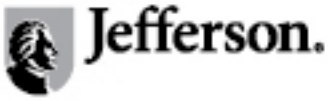

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Isoform-specific dynamic translocation of PKC by α_1-adrenoceptor stimulation in live cells

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Abstract

Protein kinase C (PKC) plays key roles in the regulation of signal transduction and cellular function in various cell types. At least ten PKC isoforms have been identified and intracellular localization and trafficking of these individual isoforms are important for regulation of enzyme activity and substrate specificity. PKC can be activated at downstream of G_q-protein coupled receptor (G_qPCR) signaling and translocated to the various cellular compartments including plasma membrane (PM). Recent reports suggested that a different type of G_qPCRs would activate different PKC isoforms (classic, novel and atypical PKCs) with different trafficking patterns. However, the knowledge of isoform-specific activation of PKC by each G_qPCR is limited. α_1-Adrenoceptor (α_1-AR) is the one of the G_qPCR highly expressed in the cardiovascular system. In this study, we examined the isoform-specific dynamic translocation of PKC in living HEK293T cells by α_1-AR stimulation (α_1-ARS). Rat PKCα, βI, βII, δ, ε and ζ fused with GFP at C-term were co-transfected with human α_{1A}-AR into HEK293T cells. The isoform-specific dynamic translocation of PKC in living HEK293T cells by α_1-ARS using phenylephrine was measured by confocal microscopy. Before stimulation, GFP-PKCs were localized at cytosolic region. α_1-ARS strongly and rapidly translocated a classical PKC (cPKC), PKCα, (< 30s) to PM, with PKCα returning diffusively into the cytosol within 5 min. α_1-ARS rapidly translocated other cPKCs, PKCβI and PKCβII, to the PM (<30s), with sustained membrane localization. One of novel PKCs (nPKCs), PKCε, but not another nPKC, PKCδ, was translocated by α_1-AR stimulation to the PM (<30s) and its membrane localization was also sustained. Finally, α_1-AR stimulation did not cause a diacylglycerol-insensitive atypical PKC, PKCζ translocation. Our data suggest that PKCα, β and ε activation may underlie physiological and pathophysiological responses of α_1-AR signaling for

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the phosphorylation of membrane-associated substrates including ion-channel and transporter proteins in the cardiovascular system.

INTRODUCTION

Protein kinase C (PKC) is a multi-gene family of serine/threonine kinase that plays key roles in the regulation of signal transduction and cellular function in various cell-types/tissues [1-4]. PKC forms a multi-gene family and at least 10 PKC isoforms have been identified that differ in primary structure, tissue distribution, subcellular localization and substrate specificity [1-4]. These isoforms can be sub-grouped into three subfamilies. Members of the first family (include PKCα, βI, βII and γ) are regulated by Ca^{2+}, diacylglycerol (DAG) and phospholipids, and are known as Ca^{2+}-dependent PKCs [or classical PKCs (cPKC)]; members of the second family (include PKCδ,ε, η and θ) have phospholipid-dependent but Ca^{2+}-independent activation mechanism, and are known as Ca^{2+}-independent PKCs or novel PKCs (nPKC); members of the third family (include PKCζ and ι/λ) are not regulated by either intracellular Ca^{2+} concentration or phospholipid and their activity are maintained by other mechanisms (e.g. by protein-protein interactions), and are known as atypical PKCs (aPKC). Intracellular localizations of these individual isoforms are important for regulation of isoform-specific enzyme activity and substrate specificity [2,3]. Since the stimulation of G_qPCRs such as α_1-adrenoceptor (α_1-AR), endothelin-1 receptor or angiotensin II receptor can generate DAG and also mobilize cytosolic Ca^{2+} elevation by releasing Ca^{2+} from the intracellular stores by stimulating inositol trisphosphate (IP_3) receptors [5], cPKC and nPKC isoforms, but not aPKC are located downstream of G_qPCR signaling pathways. Growing evidence suggests that stimulation of different G_qPCRs exhibits receptor-specific pattern of activation and translocation of PKC isoforms [3]. However, since prior research investigating PKC translocation were mostly conducted using general PKC activators, such as phorbol esters [3], the knowledge of receptor-specific regulation of PKC-isoform activation/translocation is still incomplete.

One of G_qPCRs, α_1-AR, is stimulated by catecholamines (norepinephrine and epinephrine) [6-9]. α_1-AR is expressed in variety of human tissue [10] and α_1-AR stimulation has been shown to play important roles in cellular physiological functions, such as 1) regulation of smooth muscle contraction and tone in vascular system [9], prostate, urethra, bladder [11], uterine [12] and iris [13], 2) myocardial inotropy and chronotropy [14], 3) hepatic glucose metabolism [15], 4) water secretion at salivary gland [16] and 5) neurotransmission in central nerve system [9]. In addition, chronic α_1-AR stimulation leads to pathophysiological responses in the various cells/tissues via both cPKC and nPKC isoform signaling pathways, including cardiac hypertrophy [17,18], hypertension and atherosclerosis [19,20] in cardiovascular system and portal hypertension and fibrosis in liver [21]. Despite strong interest in the mechanism underlying α_1-AR signaling-mediated pathology, especially in cardiovascular system, little is known about the molecular mechanisms for the PKC isoform-specific kinetics of activation and translocation.

Here we examined the isoform-specific dynamic translocation of PKC in live HEK293T cells by α_1-AR stimulation using GFP-tagged PKC isoforms, especially monitoring their

translocation to the plasma membrane (PM). We tested six PKC isoforms (α, βI, βII, δ, ε and ζ) in this study which are endogenously expressed in this cell line [22]. Our results show that PKCα, βI, βII and ε are able to be translocated to the PM upon α_1-AR stimulation, while PKCδ did not show any significant PM translocation upon α_1-ARS, although all isoforms are translocated to PM by a common PKC activator, phorbol 12-myristate 13-acetate (PMA) with a relatively slow time course. Our data suggest that PKCα, βI, βII and ε activation may underlie physiological responses of α_1-AR signaling. In addition, due to a prolonged retention of PKCβI, PKCβII and PKCε at PM by α_1-AR stimulation, these isoforms may be particularly important for the cellular pathophysiology in various cell types including cardiovascular system during chronic α_1-AR stimulation.

MATERIAL AND METHODS

An expanded Material and methods section is available in the online supplementary file.

Live cell imaging

HEK293T cells and stable HEK293T cell line [23] carrying HA-tagged α_{1A}-AR (Supplementary Fig.1) were transfected with GFP-tagged and/or DsRed2-tagged PKC isoforms and used for experiments 48 hours after transfection [24,25]. Time-dependent changes in localization of PKC isoforms by either phenylephrine (Phe) or PMA stimulation in HEK293T cells was measured using laser scanning confocal microscopy (Olympus, Tokyo Japan) without fixation at room temperature [25,26]. PM localization of PKC isoform was quantified by line scan intensity measurements through each cell beginning in the cytosol region (avoiding the nucleus area) and ending at the cell periphery. Translocation of each PKC isoform was evaluated by fluorescence intensity ratio between membrane and cytosolic region (membrane/cytosol ratio: M/C ratio) [25,26].

Statistical Analysis

All results are shown as mean ± standard error (SE). One-way ANOVA followed by Dunnet's test (for multiple comparison) and paired-T-test (for two-group comparison) were done with the significance level set at $P<0.05$.

RESULTS

PKCα transiently translocates to the PM in response to α_1-ARS

We co-expressed a GFP-tagged Ca^{2+}-dependent PKC isoform PKCα with α_{1A}-AR in HEK293T cells, stimulated the cells with a specific α_1-AR agonist 100 μM Phe, and observed time-dependent changes in the subcellular localization of PKCα upon Phe stimulation (Fig.1A). Prior to stimulation (0 min), PKCα-GFP mainly localized in cytosol, with no nuclear localization (Fig.1A and B). After Phe stimulation PKCα-GFP rapidly translocated to the cell membrane (less than 30s) and gradually returned to cytosol. PKCα localization was restored within 5 min despite continuous Phe stimulation. This PKC translocation was completely blocked by the α_1-AR-selective antagonist, 1 μM prazosin (Fig.1B and Supplementary Fig.2). We also tested the effect of PKC activator PMA on

PKCα-GFP translocation. PKCα-GFP showed relatively slower translocation to PM by PMA treatment compared to Phe (Fig.1B and Supplementary Fig.2).

As a control experiment, we co-expressed GFP with α_{1A}-AR in HEK293 cells and confirmed that GFP localization did not alter by Phe stimulation (Supplementary Fig.3).

PKCβI and PKCβII translocate to the PM in response to α_1-ARS

We next co-expressed other Ca^{2+}-dependent PKC isoforms (GFP-tagged PKCβI or PKCβII) with α_{1A}-AR in HEK293T cells and observed time-dependent changes in the subcellular localization of each PKC isoform by Phe (Fig.1C to F). Prior to stimulation, both PKCβI and PKCβII were localized in the cytosol, with no nuclear localization. Phe stimulation induced translocation of both PKCβI and PKCβII to the PM rapidly (after 30 sec) and PM localization was maintained throughout the course of the Phe application. Both PKCβI-GFP and PKCβII-GFP showed slower translocation to the PM by a PKC activator PMA treatment compared to Phe stimulation (Fig.1D and F), which is a similar time course as the one observed in PKCα (see Fig.1B). Interestingly, magnitude of PKCβII translocation by Phe stimulation was larger than that of PKCβI (Fig.1D and F).

To confirm the different translocation dynamics of PKCβI and βII by either Phe or PMA, we stimulated cells expressing both GFP- and DsRed2-tagged PKCβs. To normalize the magnitude of signal inputs (namely receptor signals via the G_q-protein) between the individual cells, HEK293T cells stably overexpressing α_{1A}-AR (α_{1A}-AR-HEK293T cells) were used for these experiments (Supplementary Fig.1). First, we confirmed that GFP- and DsRed2-tagged PKCβII showed similar translocation profiles under Phe or PMA stimulations in α_{1A}-AR-HEK293T (Fig.1G and H). Using α_{1A}-AR-HEK293T cells co-expressing PKCβI-GFP and DsRed2-PKCβII, we next confirmed that magnitude of PKCβII translocation by Phe stimulation was significantly larger than that of PKCβI (Fig.1I and J). In contrast, PMA stimulation induces larger translocation of PKCβI than that of PKCβII (Fig.1J).

PKCε but not PKCδ translocate to the PM in response to α_1-ARS

We next tested whether Ca^{2+}-independent PKC isoforms translocates to the PM by α_1-ARS. We co-expressed GFP-tagged PKCδ or PKCε with α_{1A}-AR in HEK293T cells and observed time-dependent changes in the subcellular localization of these PKC isoforms upon Phe application. Prior to stimulation, PKCδ was localized in cytosol (Fig.2A). Some cells have strong punctuated fluorescence areas around the nucleus (Supplementary Fig.4). No significant time-dependent changes were observed in the subcellular localization of PKCδ by Phe treatment (Fig.2A and B). As a positive control, we observed significant translocation of PKCδ in response to treatment with PMA in the same cells (Fig2A and B). After PMA treatment, PKCδ was translocated from the cytosol to the PM and also to the nuclear membrane (Fig.2A, 2B and supplementary Fig.4). We also confirmed the lack of GFP-tagged PKCδ translocation by Phe using α_{1A}-AR-HEK293T cells co-transfected with DsRed2-PKCβII (Fig.2C). In contrast, PKCε was significantly translocated from the cytosol to the PM 3 min after Phe stimulation and its PM localization was maintained during the course of the Phe application (Fig.2D and E). Magnitude of GFP-tagged PKCε translocation

by 10-min Phe stimulation was larger than that in PKC βI and also reached to the similar level of that in PKCβII (see Fig.1D and F). To quantitatively compare the translocation levels of PKCε and PKCβI by Phe, we stimulated α_{1A}-AR-HEK293T cells expressing both GFP-PKCε and DsRed2-tagged PKCβI. The magnitude of PKCε translocation was consistently higher after Phe stimulation than that of PKCβI when measured in the same cell (Fig.2F, left). In contrast, PMA stimulation induces similar translocation profiles in these PKC isoforms (Fig.2F, right).

PKCζ does not show any translocation in response to α_1-ARS

Finally, we tested whether DAG- and PMA-insensitive isoform (aPKC) change its subcellular localization in response to α_1-ARS. We co-expressed one of the aPKC isoform PKCζ in HEK293 cells and monitored time-dependent changes in the subcellular localization of PKCζ upon Phe application. Prior to stimulation, PKCζ was localized in cytosol and no nuclear localization was observed (Fig. 3). There was no significant time-dependent change in the subcellular localization of PKCζ by α_1-ARS.

DISCUSSION

In this study, we examined the translocation profiles of PKC isoforms to the PM in live cells expressing GFP- and DsRed2-tagged PKC isoforms upon α_1-AR stimulation. One advantage of using fluorescence protein-tagged PKC expression system is the ability to obtain more precise and quantitative spatiotemporal kinetics information on PKC activation and translocation profiles compared to classical methods, including Western blotting of fractionated proteins and the immunocytochemstry of fixed cells using isoform-specific antibodies [2,3]. Our results clearly indicate that each PKC isoform shows a different spatiotemporal pattern of translocation under α_1-ARS (Fig. 4). We found the significant translocation of Ca^{2+}-dependent PKCs (α, βI and βII) and one Ca^{2+}-dependent PKC (PKCε) to the PM in response to α_1-AR stimulation, which may underlie acute responses of α_1-AR signaling at the PM. However, subcellular localization of another Ca^{2+}-dependent PKC, PKCδ was not significantly modified by α_1-ARS in the current experiments, despite showing a strong response to PMA (Fig.2).

Physiological and pathophysiological functions of each PKC isoform are diverse even though their substrate specificity is low and multiple isoforms are expressed in each cell-type [2,3]. For instance, in the human myocardium, PKCα was the most abundant isoform present [19; 20] and PKCα activation has been linked to heart failure, hypertrophy and diabetes [21-27]. However, subsequent studies in genetic models of PKCα overexpression or knockout in mice failed to yield significant role of PKCα in generating hypertrophy to PKCα, identifying a more predominant role for PKCα in the regulation of cardiac contraction [28]. On the other hand, expression level of Ca^{2+}-dependent PKCβ increases in the cardiovascular system during disease states. PKCβ activation has been linked to increased vascular inflammation and atherosclerosis [29]. In addition, genetic models showed that PKCβ expression is not necessary for the development of cardiac hypertrophy nor does its absence attenuate the hypertrophic response [30]. PKCε has been suggested to have a protective role in myocardial ischemia-reperfusion injury [31] and arrhythmias [32].

Moreover, the use of specific PKC isoenzyme blockers may lead to novel treatments for heart failure [35; 36] and also for cancers [33] and Alzheimer's disease [34]. Targeting PKC to the PM is crucial for the phosphorylation of membrane-associated substrates, including ion-channel and transporter proteins [27]. PMA is one of the well recognized activators of DAG-sensitive PKC subfamilies and is frequently used to mimic G_qPCR signaling for investigating the effect of PKC translocation [2,3] to the PM, including in the studies for the regulation of ion channel function. However, we showed in live cells that the translocation kinetics of PKCs are largely different in response to either PMA treatment or receptor stimulation. First, α_1-AR stimulation by Phe initiates PKC translocation much faster than PMA started ($\approx$ 30 sec after stimulation). Second, while G_qPCR-mediated PKCα translocation to the PM is transient, PMA treatment induces this isoform to be retained at the PM (Figs.1 and 4). Finally, isoform-specificity is also different between G_qPCR and PMA responses; PMA caused sustained translocation of all DAG-sensitive isoforms tested to the PM (Figs.1 and 2), while α_1-AR-mediated PKCδ translocation to the PM was not observed (Fig.2). This observation is consistent with previous data showing that connexin43, a gap junction channel protein, can be phosphorylated by PMA treatment through both PKCδ and PKCε [28,29], but PKCδ does not likely phosphorylate Connexin43 under G_qPCR stimulation [30]. Thus, these results indicate that great caution is required when extending the results obtained with general PKC activators to PKC isoform activation by more physiological signaling pathways.

While we did not observe significant PKCδ translocation by α_1-AR stimulation from cytosol to the PM using transfected PKCδ-GFP, our data does not exclude the possibility that PKCδ can be activated by α_1-AR stimulation and translocated to the other cellular compartments including mitochondria and nucleus. For instance, Newton and colleagues detected a relatively small but significant PKCδ translocation to the outer mitochondrial membrane (OMM) after the application of a phorbol ester using fluorescence resonance energy (FRET) by generating an OMM-targeted [31] CFP (mt-CFP) and PKCδ-GFP [32]. They also confirmed that PKCδ activity was increased at the OMM upon phorbol-ester stimulation, using the OMM-targeted FRET-based PKCδ kinase activity reporter. Taken together, the data suggest that G_qPCR stimulation may activate and translocate PKCδ to other intracellular compartments, but not to PM.

In summary, we showed that α_1-AR induces PKCα, PKCβ and PKCε translocation to the PM, but not PKCδ and PKCζ by tracking the subcellular localization of transfected GFP-tagged PKC after Phe stimulation. In addition, we found that kinetics of α_1-AR-mediated activation of PKCs is isoform-specific. Our data suggests that activation of PKCα, PKCβ and PKCε may underlie physiological and pathophysiological responses of α_1-AR signaling for the phosphorylation of PM-localized proteins such as ion channels and transporters.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

α_1-AR	α_1-adrenoceptor
α_{1A}-AR-HEK293T cells	HEK293T cells stably overexpressing α_{1A}-AR
aPKC	atypical protein kinase C
cPKC	classical protein kinase
DAG	diacylglycerol
nPKC	novel protein kinase
Phe	phenylephrine
G_qPCR	G_q-protein coupled receptor
M/C ratio	membrane/cytosol ratio
PKC	protein kinase C
PM	plasma membrane

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Highlights

- Isoform-specific translocation pattern of PKC was observed in live cells.
- PKCα, βI, βII, and ε translocates to plasma membrane by α_1-adrenergic stimulation.
- PKCδ did not show any translocation to plasma membrane by α_1-adrenergic stimulation.
- PKC translocation kinetics were different between PMA and α_1-adrenergic stimulation.
- PKCα, βI, βII and ε may underlie physiological responses of α_1-adrenergic signaling.

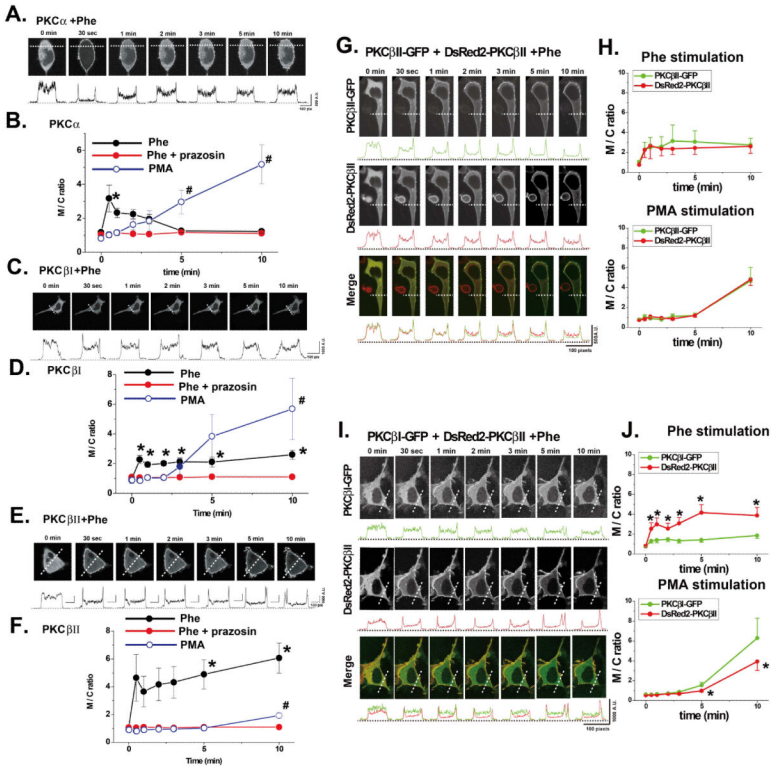

Fig.1. Ca^{2+}-dependent PKC isoforms translocate to the PM in response to α_1-ARS

A. Representative cell images (top) and fluorescence intensity profiles at white dot lines (bottom) in cells expressing α_{1A}-AR and PKCα-GFP during Phe stimulation. A.U., fluorescence arbitrary units. **B.** Time-dependent changes in the subcellular localization of PKCα-GFP by Phe in the presence or in the absence of α_1-AR antagonist prazosin. The effect of PMA is also shown. *p<0.05, compared to control (0 min, before Phe stimulation). #p<0.05, compared to control (0 min, before PMA stimulation). **C.** Representative cell images (top) and fluorescence intensity profiles (bottom) in cells expressing α_{1A}-AR and PKCβI-GFP during Phe stimulation. **D.** Time-dependent changes in the subcellular localization of PKCβI-GFP by Phe in the presence or in the absence of α_1-AR antagonist prazosin. The effect of PMA is also shown. *p<0.05, compared to control (0 min, before Phe stimulation). #p<0.05, compared to control (0 min, before PMA stimulation). **E.** Representative cell images (top) and fluorescence intensity profiles (bottom) in cells expressing α_{1A}-AR and PKCβII-GFP during Phe stimulation. **F.** Time-dependent changes in the subcellular localization of PKCβII-GFP by Phe in the presence or in the absence of α_1-AR antagonist prazosin. The effect of PMA is also shown. *p<0.05, compared to control (0 min, before Phe stimulation). #p<0.05, compared to control (0 min, before PMA stimulation). **G.** Representative cell images and fluorescence intensity profiles in α_{1A}-AR-HEK293T cells co-expressing PKCβII-GFP and DsRed2-PKCβII during Phe stimulation. **H.** Time-dependent changes in the subcellular localization of PKCβII-GFP and DsRed2-PKCβII by Phe (top) or PMA (bottom) in α_{1A}-AR-HEK293T cells. **I.** Representative cell images and fluorescence intensity profiles in α_{1A}-AR-HEK293T cells co-expressing PKCβI-GFP and DsRed2-PKCβII during Phe stimulation. **J.** Time-dependent changes in the subcellular localization of PKCβI-GFP and DsRed2-PKCβII by Phe (top) or

PMA (bottom) in α_{1A}-AR-HEK293T cells. *$p<0.05$, compared to PKCβI-GFP at each time point.

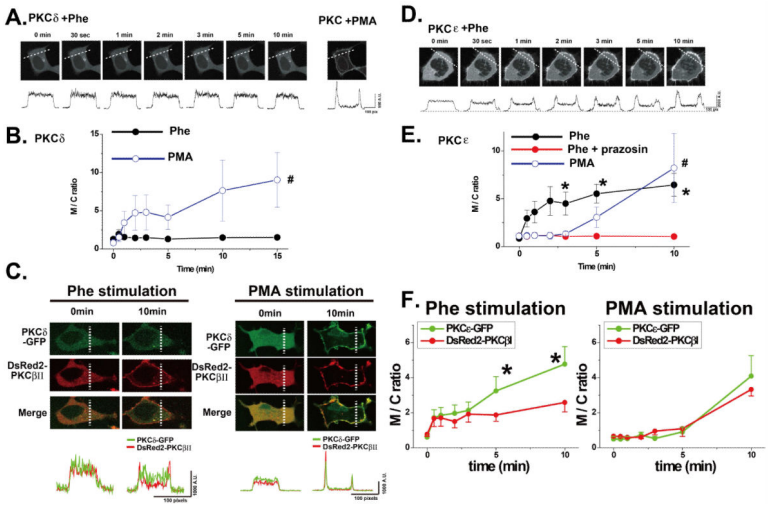

Fig.2. Ca^{2+}-independent PKC, PKCε does (but PKCδ does not) translocate to the plasma membrane in response to α_1-ARS

A. Representative cell images (top) and fluorescence intensity profiles at white dot lines (bottom) in cells expressing α_{1A}-AR and PKCδ-GFP during Phe stimulation. PKC δ translocation by PMA treatment was also observed in same cell as a positive control (right) (see also Supplementary Fig.4). A.U., fluorescence arbitrary units. **B.** Time-dependent changes in the subcellular localization of PKCδ-GFP by the treatment of Phe or PMA. $^{\#}p<0.05$, compared to control (0 min, before PMA stimulation). **C.** *Left*, representative cell images (top) and fluorescence intensity profiles at white dot lines (bottom) in α_{1A}-AR-HEK293T cells co-transfected with PKCδ-GFP and DsRed2-PKCβII before and after Phe stimulation. The results shown are representative of five cells. *Right*, representative cell images (top) and fluorescence intensity profiles at white dot lines (bottom) in α_{1A}-AR-HEK293T cells co-transfected with PKCδ-GFP and DsRed2-PKCβII before and after PMA stimulation. The results shown are representative of six cells. A.U., fluorescence arbitrary units. **D.** Representative cell images (top) and fluorescence intensity profiles at white dot lines (bottom) in cells expressing α_{1A}-AR and PKCε-GFP during Phe stimulation. **E.** Time-dependent changes in the subcellular localization of PKCε-GFP by 100 μM Phe in the presence or in the absence 1 μM prazosin. The effect of PMA is also shown as a positive control. $^{*}p<0.05$, compared to control (0 min, before Phe stimulation). $^{\#}p<0.05$, compared to control (0 min, before PMA stimulation). **F.** Time-dependent changes in the subcellular localization of PKCε-GFP and DsRed2-PKCβI by Phe (left) or PMA (right) in α_{1A}-AR-HEK293T cells. $^{*}p<0.05$, compared to DsRed2-PKCβI at each time point.

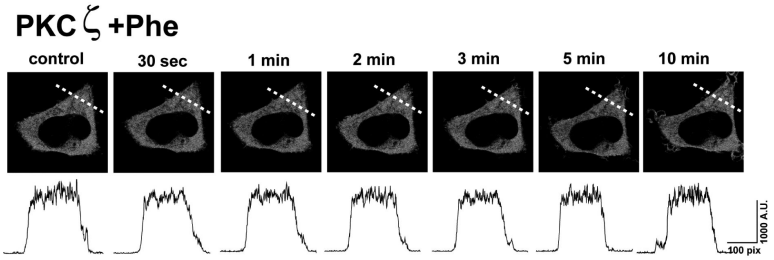

Fig.3. Atypical PKC, PKCζ does not show any translocation by α_1-ARS

Representative cell images (top) and fluorescence intensity profiles at white dot lines (bottom) in cells expressing α_{1A}-AR and PKCζ-GFP during α_1-ARS (100 μM Phe). The results shown are representative of four cells. A.U., fluorescence arbitrary units.

A.

	localization at rest	translocation by Phe	translocation by PMA
α	cytosol	Yes (Plasma membrane)	Yes (Plasma membrane)
βI	cytosol	Yes (Plasma membrane)	Yes (Plasma membrane)
βII	cytosol	Yes (Plasma membrane)	Yes (Plasma membrane)
δ	cytosol (Golgi?)	No	Yes (Plasma membrane and nuclear membrane)
ε	cytosol	Yes (Plasma membrane)	Yes (Plasma membrane)
ζ	cytosol	No	No
GFP	cytosol and nuclear	No	No

B.

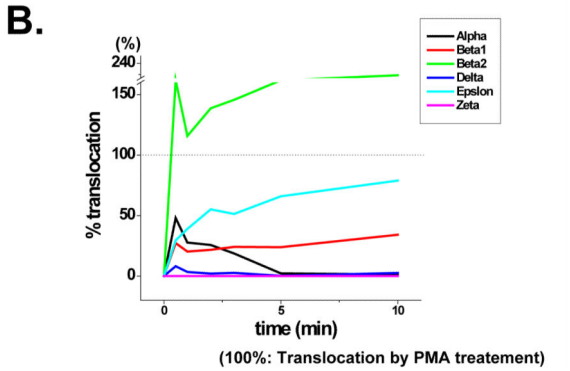

Fig.4. Isoform-specific translocation of PKC by α_1-ARS in HEK293Tcells

A. Summary table of the Isoform-specific translocation of PKC by α_1-ARS (Phe treatment) or PMA treatment in HEK293Tcells. **B.** Summary of the time-dependent translocation of PKC isoforms to the PM by α_1-ARS in HEK293T cells. M/C ratio in each isoform was normalized by that obtained after 10-min application of PMA (set as a 100% translocation).