

Spatiotemporal Analysis of the Molecular Interaction between PICK1 and PKC

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PICK1 is a protein which was initially identified as a protein kinase C α (α PKC) binding protein using the yeast two-hybrid system. In addition to α PKC, the PICK1 complex binds to and regulates various transmembrane proteins including receptors and transporters. However, it has not been clarified when and where PICK1 binds to α PKC. We examined the spatiotemporal interaction of PICK1 and PKC using live imaging techniques and showed that the activated α PKC binds to PICK1 and transports it to the plasma membrane. Although the membrane translocation of PICK1 requires the activation of α PKC, PICK1 is retained on the membrane even after PKC moves back to the cytosol. These results suggest that the interaction between α PKC and PICK1 is transient and may not be necessary for the regulation of receptors/transporters by PICK1 or by α PKC on the membrane.

Key words: protein kinase C, PICK1, PDZ, translocation, phosphorylation

I. Introduction

Protein kinase C (PKC) plays a pivotal role in many signaling pathways and the existence of multiple subtypes suggests that different isoforms have various functions. The PKC superfamily consists of at least 10 subtypes and is divided into three groups, classical PKC (cPKC; α , β I, β II, γ), novel PKC (nPKC; δ , ϵ , η , θ), and atypical PKC (aPKC; ζ , ι/λ), based on their structures [20, 21, 25, 26]. Each PKC subtype has differential sensitivity to activators and shows tissue- and cell-specific expression, suggesting that each subtype plays a subtype-specific role in various signal transduction pathways and in the regulation of numerous cellular processes [20–22]. The subtype-specific function of PKC, however, has not been clarified by conventional biochemical techniques due to the low substrate specificity among family members. Recent technical developments enabled us to monitor the movement of PKC in living cells using PKC fused with green fluorescent protein (GFP) [23, 29]. Reports using GFP-tagged PKC subtypes have demonstrated that PKC translocation varies depending on the PKC subtype and on the kind of stimulation [1, 15, 18, 19, 23, 32,

39, 40]. These results suggest that the correct spatiotemporal translocation of each PKC subtype is necessary for its activation and phosphorylation of the specific substrates. Analysis of subtype- and stimulus-specific PKC translocation provides a way to study their individual role in cell signaling pathways.

A characteristic and important feature of central nervous system synapses is the clustering of receptors and signaling molecules at both postsynaptic and presynaptic sites, which underlies an efficient synaptic transmission in the brain. For instance, at the glutamate excitatory synapses of the hippocampus, both ionotropic glutamate *N*-methyl-D-aspartate (NMDA) and α -amino-3-hydroxy-5-methylsoxazole-4-propionate (AMPA) receptors are concentrated at postsynaptic sites [36], whereas the G protein-coupled metabotropic glutamate receptor mGluR7a is specifically targeted to the presynaptic active zone of nerve terminals [31]. Recent studies have shown the importance of PDZ domain-containing proteins in mediating the specific synaptic clustering of neurotransmitter receptors and in regulating receptor signaling at synapses [5, 10, 11].

Among the protein interacting with protein kinase C (PKC), PICK1 is a PDZ domain protein originally cloned on the basis of its interaction with PKC by a yeast two-hybrid screening [33]. PICK1 selectively binds to α PKC through interaction with the QSAV sequence at the extreme COOH-terminus of α PKC [34]. The carboxylate-binding

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loop within the PDZ domain of PICK1 is required for the interaction [34]. PICK1 has subsequently been shown to interact with the Eph receptor tyrosine kinases and ephrin-B ligands [37], GluR2/3/4c subunits of the AMPA receptor [6, 41], mGluR7a receptor [3, 7], and dopamine and norepinephrine transporters [38]. PICK1 forms a triple complex containing α PKC and mGluR7 in neurons [7].

In the present study, we examined the spatio-temporal interaction between PICK1 and α PKC in cells by live imaging techniques to elucidate the functional role of PKC as a binding partner for PICK1.

II. Materials and Methods

Materials

12-*O*-tetradecanoylphorbol 13-acetate (TPA), PKC inhibitor (Gö6983), and uridine triphosphate (UTP) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade.

Cell culture

HEK293 cells were purchased from Riken Cell Bank (Tsukuba, Japan). CHO-K1 cells were purchased from Health Science Research Resources Bank (Osaka, Japan). HEK293 cells were cultured in Dulbecco's modified Eagle's medium, and CHO-K1 cells in Ham's F-12 medium (Invitrogen, Grand Island, NY) at 37°C in a humidified atmosphere containing 5% CO₂. All media were supplemented with 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 µg/ml). The fetal bovine serum used was not heat-inactivated. For transfection experiments, HEK293 cells and CHO-K1 cells were trypsinized and seeded at a density of 1×10⁵ cells/3.5-mm on glass-bottomed culture dishes (MatTek Corp., Ashland, MA) and incubated for 16–24 hr before transfection.

Transfection of plasmids into cultured cells

CHO-K1 and HEK293 cells were transfected using 3 µl of FuGENETM 6 Transfection Reagent (Roche Molecular Biochemicals) and 1 µg of DNA according to the manufacturer's protocol. For co-expression of plasmids, the same amount of each plasmid was mixed and transfected. Experiments were performed 24–48 hr after the transfection.

Construction of plasmids encoding the DsRed2-PKCs and GFP-PICK1 fusion protein

BS1043 was a plasmid encoding DsRed2- α PKC fusion protein in which α PKC (BS611) and DsRed2 were bound at the N-terminus of α PKC. BS1085 was a plasmid encoding DsRed2- γ PKC fusion protein in which γ PKC in pTB701 (BS55) and DsRed2 were bound at the N-terminus of γ PKC. BS1104 was a plasmid encoding DsRed2- γ PKC-CT α fusion protein. A cDNA fragment of γ PKC in pTB701 (BS55) with a HindIII site in the 5'-terminus and an EcoRI site in the 3'-terminus was produced by a PCR with cDNA for γ PKC in pTB701 (BS55) as the template. The sense and antisense primers were 5'-TTAAGC TTCCATGGCG-

GGTCTGGGTCTG-3' and 5'-TTGAATTCTCATACTG-CACTTTGCA AGATTGGGTGCACGAAGTCCGG GTT-CAC-3', respectively. The PCR product of γ PKC was first subcloned into a TA cloning vector, pCRTM2.1 (Invitrogen, San Diego, CA). The plasmid was digested with HindIII and EcoRI then subcloned into the HindIII and EcoRI site in pDsRed2-C1 (Clontech Laboratories, Inc). GFP were bound at the N-terminus of PICK1 to produce GFP-PICK1 (BS 1001). Plasmids encoding GFP-PICK1-T82A, -T227A and -T249A) was constructed by the mutation of each threonine to alanine.

Observation of translocation of GFP- or DsRed2-fused protein

Transfected cells were spread onto glass-bottomed culture dishes (MatTek) and cultured for at least 24 hr before observation. The culture medium was replaced with normal HEPES buffer composed of 135 mM NaCl, 5.4 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, and 10 mM glucose, pH 7.3. The fluorescence of the fusion proteins was monitored by confocal laser scanning fluorescence microscopy (model LSM 510 invert; Carl Zeiss, Jena, Germany). GFP-fused protein was monitored at 488-nm argon excitation using a 510- to 535-nm band pass barrier filter. DsRed-fused protein was monitored at 543-nm HeNeI excitation using a 590-nm-band pass barrier filter. DsRed and GFP were monitored simultaneously using multitracking software which alternately detects each fluorescence by switching quickly between laser and filter system.

Immunoprecipitation

GFP- α PKC cDNA was transiently transfected into HEK293 cells (1×10⁵ cells/dish) by FuGENETM 6 Transfection Reagent (Roche, Mannheim). After transfected cells were cultured at 37°C for 16 hr, and then harvested with 5 ml of homogenate buffer (250 mM sucrose, 10 mM EGTA, 2 mM EDTA, 20 mM Tris-HCl, 20 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4) containing 1% Triton-X 100. After the sonication (UD-210 Tomy Seiko, Tokyo, Japan) (output, 5; duty, 50%; 10 times at 4°C), samples were centrifuged at 400,000×g for 30 min at 4°C (Hitachi, Tokyo, Japan), and supernatant was collected. The supernatant was rotated with an anti-GFP polyclonal antibody (diluted 1:50) (Molecular Probes, Eugene, OR) for 2 hr at 4°C and then with protein A-Sepharose for an additional 2 hr. Samples were centrifuged at 2,000×g for 5 min at 4°C, and pellets were washed three times with phosphate-buffered saline without Ca²⁺ and Mg²⁺ (PBS(-)). Finally, the pellet was suspended in 50 µl of PBS(-) and used for kinase assays or immunoblot.

Kinase assay of recombinant GFP- α PKC

GFP- α PKC was immunoprecipitated from HEK293 cells transfected with GFP- α PKC cDNA. The immunoprecipitated samples (10 µl of suspended pellet) were used for in vitro kinase assays. The kinase activity was assayed by measuring the incorporation of ³²Pi into MBP from

[γ - 32 P]ATP as described previously [24].

Data analysis

The time course of translocation was recorded as a time series of 30–50 images for each experiment. Image analysis was performed using the Zeiss LSM 510 software, and the membrane fluorescence ratio was calculated. This was defined as the plasma membrane fluorescence intensity/cytoplasm fluorescence intensity in 1–7 μm^2 region of interest (ROI). ROIs were circles for cytoplasm and rectangles for the plasma membrane. For each time point, the membrane fluorescence was calculated from at least 5 different ROIs. These values were averaged and plotted to generate a time course of translocation.

III. Results

Intense fluorescence of DsRed2- α PKC was observed in the perikarya of the transfected HEK293 cells which express DsRed2- α PKC alone, and faint fluorescence was

seen in the nuclei (Fig. 1A lower row). The treatment with 1 μM TPA induced the obvious translocation of DsRed2- α PKC from the cytosol to the plasma membrane. The translocation began at 5 min and was completed by 15 min after TPA stimulation (Fig. 1A). The fluorescence remained on the plasma membrane for at least 25 min after TPA treatment and did not return to the cytoplasm in the cells tested. The DsRed2- α PKC in the nuclei did not appear to be translocated. Intense fluorescence of GFP-PICK1 was observed in the perikarya of the transfected HEK293 cells which express GFP-PICK1 alone, and faint fluorescence was seen in the nuclei (Fig. 1A upper row). In contrast, the fluorescence of GFP-PICK1 did not translocate after 1 μM TPA stimulation.

Then we investigated the translocations of PKC and PICK1 when co-expressed in the same cells. Co-expression of DsRed2- α PKC with GFP-PICK1 did not alter the diffuse distribution of each fluorescent protein (Fig. 1B). When the cells were stimulated with 1 μM TPA, both GFP-PICK1 and DsRed2- α PKC translocated from the cytosol to the

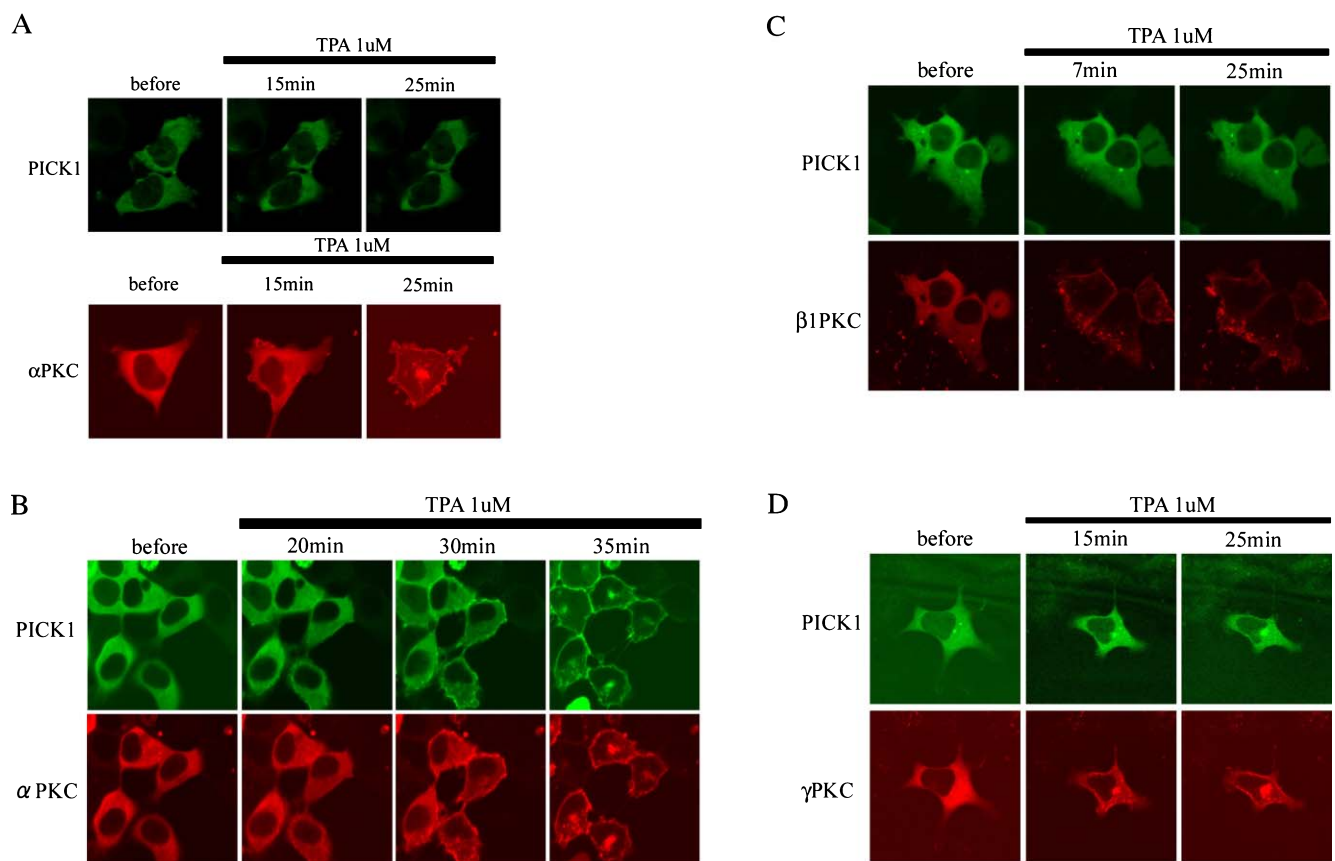


Fig. 1. **A:** The translocation of GFP-PICK1 or DsRed2- α PKC, when expressed alone in HEK293 cells. GFP-PICK1 was observed throughout the cytoplasm in transfected HEK293 cells. The fluorescence of GFP-PICK1 was not translocated by 1 μM TPA at 37°C. In contrast, 1 μM TPA induced the obvious translocation of DsRed2- α PKC from the cytosol to the plasma membrane. **B:** TPA-induced translocation of GFP-PICK1 when co-expressed with DsRed2- α PKC in HEK293 cells. Both GFP-PICK1 and DsRed2- α PKC were observed throughout the cytoplasm in transfected HEK293 cells. Activation of PKC by 1 μM TPA induced the obvious translocation of GFP-PICK1 and DsRed2- α PKC. Translocation was completed within 20 min after the treatment with TPA. **C & D:** TPA-induced translocation of GFP-PICK1 when co-expressed with DsRed2- β 1PKC (**C**), or DsRed2- γ PKC (**D**). GFP-PICK1 fusion protein was not translocated by TPA when co-expressed with β 1PKC or γ PKC.

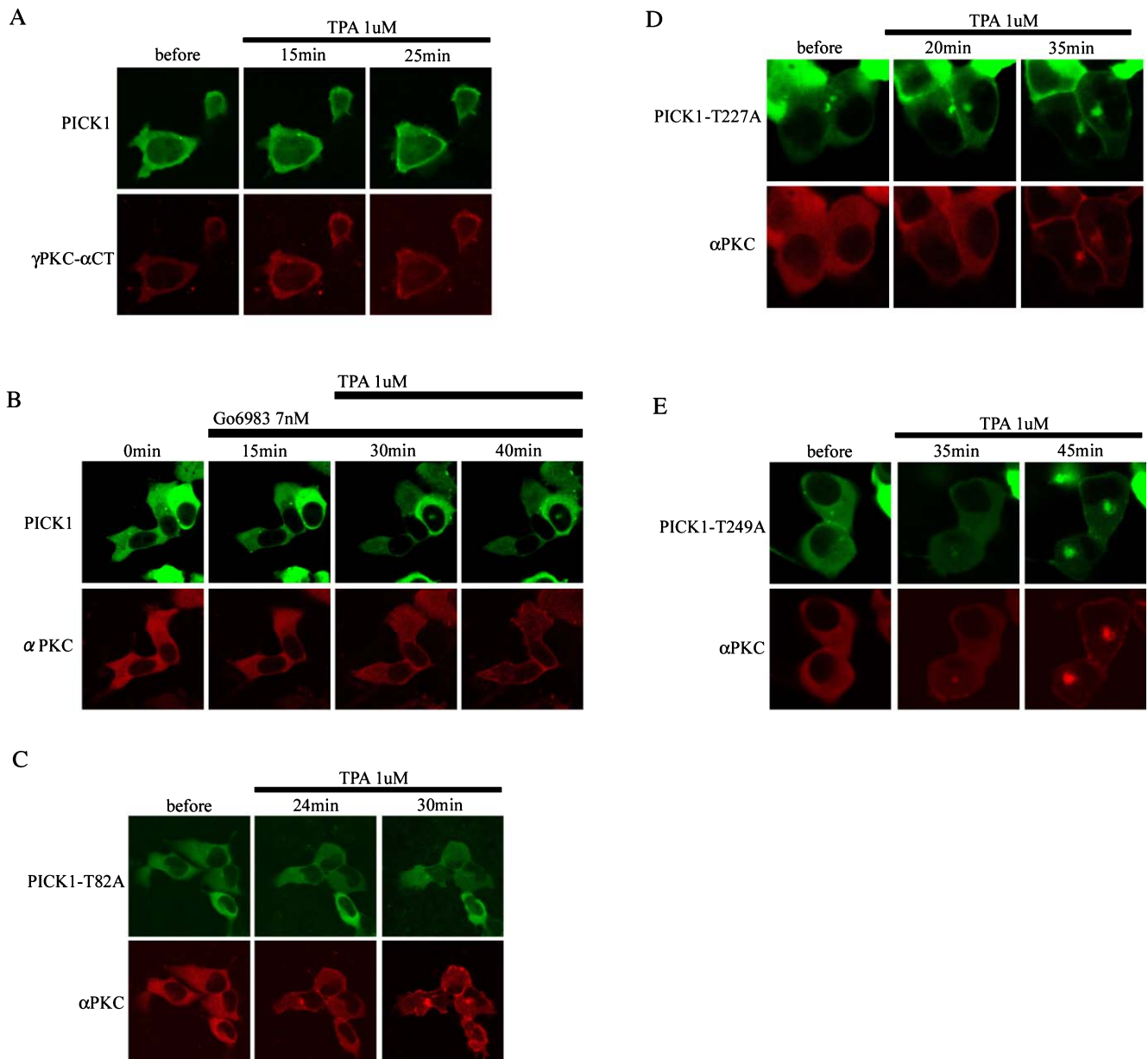


Fig. 2. **A:** Role of C-terminal region of α PKC for TPA-induced translocation of PICK1 in HEK293 cells. Activation of PKC by 1 μ M TPA induced the obvious translocation of GFP-PICK1 and DsRed2- γ PKC which had the C-terminal region of α PKC (DsRed2- γ PKC-CT α) from the cytosol to the plasma membrane. **B:** Effects of α PKC activity on PICK1 translocation. Pretreatment with 7 nM G δ 6983 (37°C for 15 min) abolished the TPA-induced translocation of GFP-PICK. **C:** Effect of T82A mutation of PICK1 on its translocation. Substitution of threonine82 of PICK1 to alanine abolished TPA-induced translocation of the mutant PICK1 but not on that of α PKC. **D:** Effect of T227A mutation of PICK1 on its translocation. Substitution of threonine227 of PICK1 to alanine did not alter either the TPA-induced translocation of the mutant PICK1 or α PKC. **E:** Effect of T249A mutation of PICK1 on its translocation. Substitution of threonine249 of PICK1 to alanine did not alter either the TPA-induced translocation of the mutant PICK1 or α PKC.

membrane within 15–20 min after the stimulation (Fig. 1B), although GFP-PICK did not respond to TPA when expressed alone. These results suggest that translocation of GFP-PICK1 was induced by the activation and translocation of exogenous α PKC.

Staudinger *et al.* reported that PICK1 interacts with the PDZ binding site in the C terminus of α PKC through a PDZ

domain within PICK1 [33, 34]. To elucidate the subtype-specificity of PKC for interaction with PICK1, we examined the translocation of GFP-PICK1 in the cells which simultaneously express γ PKC or β IPKC. As shown in Figure 1C and 1D, TPA failed to translocate GFP-PICK1 to the plasma membrane, while the TPA-induced translocation of γ PKC or β IPKC was evident, suggesting that the translocation of

PICK1 is related with the expression of α PKC but not γ PKC or β PKC. As it was supposed that α PKC binds to PICK1 through its C terminal with PDZ domain of PICK1, the binding domain of α PKC to translocate with PICK1 was further examined by using γ PKC having C terminal of α PKC. The four amino acid residues at the C terminal of α PKC have been shown as a PDZ-binding site. The last four amino acids of γ PKC were substituted with PDZ-binding sequence of α PKC (γ PKC- α CT) and co-expressed with PICK1. As shown in Figure 2A, the simultaneous translocation of GFP-PICK1 with γ PKC- α CT to the plasma membrane was observed after TPA treatment.

To study whether not only the binding of α PKC to PICK1 but also the phosphorylation of PICK1 by α PKC is necessary for the TPA-induced translocation of PICK1, we examined the effect of a PKC inhibitor (Gö6983) on the PICK1 translocation in the HEK293 cells expressing GFP-PICK1 and DsRed2- α PKC (Fig. 2B). In the presence of 7 nM Gö6983, neither the cytosolic localization of PICK1 nor that of α PKC was altered. Gö6983, however, blocked the translocation of GFP-PICK1, while DsRed2- α PKC showed membrane translocation. The results indicate that the TPA-induced translocation of PICK1 requires both the α PKC activation in addition to the binding of α PKC to PICK1.

Three potential PKC phosphorylation sites, R/KXXS/T or R/KXS/T were found in the amino acid sequence of PICK1 [27]. We further studied whether or not PICK1 is phosphorylated by α PKC after its binding to active α PKC and whether or not the phosphorylation is necessary for the TPA-induced translocation of PICK1. We constructed three mutants of PICK1 (PICK1-T82A, T227A, T249A) in which potential PKC phosphorylation sites was mutated and examined their translocation in response to TPA. Mutation of threonine 82 to alanine (T82A) of PICK1 blocked the translocation of PICK1 by TPA (Fig. 2C), although PICK1-T227A (Fig. 2D) and PICK1-T249A (Fig. 2E) showed evident membrane translocation. Translocation of α PKC was evident after TPA treatment in all HEK293 cells expressing these PICK1 mutants. These results suggested that the TPA- and α PKC-dependent translocation of PICK1 to the plasma membrane requires the phosphorylation of threonine 82 (T82).

As shown above, TPA-induced translocation of α PKC was slow and irreversible. Therefore, it is difficult to detect the temporal difference of the translocation between α PKC and PICK1. We used UTP stimulation to translocate α PKC, which induces a rapid and reversible translocation [32]. Activation of α PKC through G protein-coupled receptor stimulation induced rapid and reversible translocation of both DsRed2- α PKC and GFP-PICK1 but the time course of the membrane targeting differed between DsRed2- α PKC and GFP-PICK1 (Fig. 3). The temporal difference of the two translocations is shown in Figure 3A. The fluorescence of DsRed2- α PKC translocated from the cytosol to the membrane within 30 sec and redistributed from the membrane to the cytosol within 150 sec. In contrast, the translocation of GFP-PICK1 was evident at 60 sec, still localized on the

membrane at 150 sec and redistributed from the membrane to the cytosol within 300 sec after stimulation with 100 μ M UTP. The intensity of the fluorescence on the membrane and in the cytoplasm was measured and the membrane/cytosol ratio of the fluorescence was quantified (Fig. 3B). Figure 3B shows that the membrane translocation of α PKC precedes that of PICK1, and that PICK1 is retained on the membrane longer than α PKC. These findings suggested that once PICK1 is targeted to the plasma membrane by the association with the activated α PKC, it is not necessary for PICK1 to associate with α PKC.

The relation between the kinase activity of α PKC and its binding to PICK1 was further analyzed (Fig. 4). The time course of PKC activity was assayed by measuring the incorporation of 32 Pi from [γ - 32 P]ATP into MBP at each time point after the addition of the activators, PS/DO/ Ca^{2+} as described in Materials and Methods. α PKC was significantly activated 10 sec after the treatment with PS/DO/ Ca^{2+} and the kinase activity of α PKC was progressively increased at least until 180 sec. However, pulldown assay using anti-GFP antibody showed that the maximal interaction of PICK1 with GFP- α PKC was found 60 sec after the activation of GFP- α PKC, while the interaction was very faint before the stimulation. Interestingly, the interaction was decreased at 180 sec when the kinase activity was still increasing. This strongly suggested that PICK1 does not bind to α PKC continuously even when α PKC is in active state.

IV. Discussion

The present study investigated the detailed mechanism of PKC-dependent translocation of PICK1 in living cells. TPA did not translocate exogenous PICK1, when PICK1 alone was overexpressed in HEK293 cells, while PICK1 was translocated when co-expressed with α PKC. PICK1 translocated to the plasma membrane within 30 min in response to TPA as α PKC was translocated. This PKC-dependent translocation of PICK1 was α PKC-specific, although other PKC subtypes such as β PKC and γ PKC showed TPA-induced translocation similar to that of α PKC. It is suggested that the subtype specific interaction of α PKC with PICK1 is caused by the binding of PDZ domain of PICK1 to the PDZ-binding site (QSAV) at the COOH terminus of α PKC [34]. Indeed, TPA-induced translocation of PICK1 to the plasma membrane was observed when γ PKC which has a QSAV sequence at C-terminal was co-expressed, indicating that the PDZ-binding sites of α PKC, QSAV, is necessary for PKC-dependent translocation of PICK1. However, TPA failed to translocate PICK1 without overexpression of α PKC, although α PKC is expressed endogenously in HEK293 cells. Significant amounts of α PKC are perhaps necessary for the evident translocation of GFP-tagged PICK1.

TPA-induced translocation of PKC was first visualized by using GFP-tagged PKCs in living cells [29]. TPA induced slow and irreversible translocation of PKC and the translocation did not depend on the PKC activity as PKC

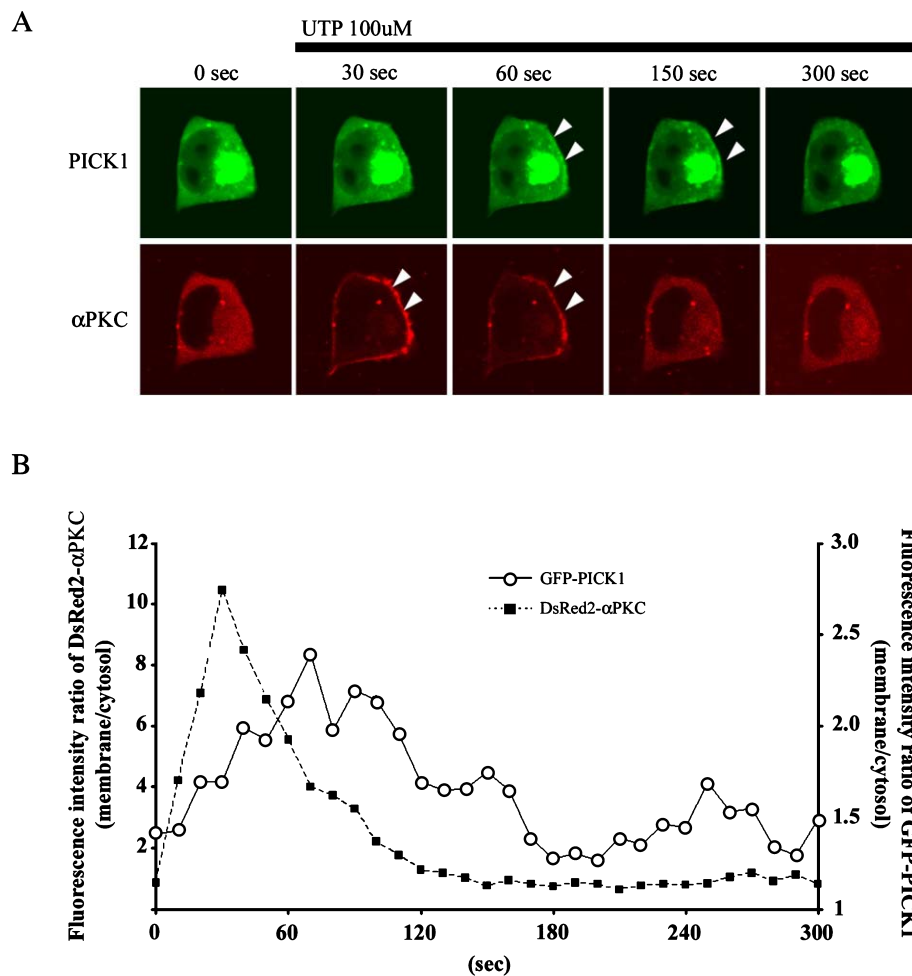


Fig. 3. **A:** Simultaneous observation of UTP-induced translocation of GFP-PICK1 and DsRed2- α PKC. Change in the fluorescence of GFP-PICK1 and DsRed2- α PKC expressed in CHO-K1 cells by 100 μ M UTP at 37°C. Activation of PKC by 100 μ M UTP induced the obvious translocation of DsRed2- α PKC fluorescence from the cytosol to the plasma membrane within 30 sec and retranslocation to the cytosol was seen within 120 sec. In contrast, the GFP-PICK1 moved to the plasma membrane at 60 sec and reversed to the cytosol at 180 sec. **B:** Membrane/cytosol ratio of GFP-PICK1 and DsRed2- α PKC after UTP stimulation. Membrane/cytosol ratio of DsRed2- α PKC peaks at 30 sec, while that of GFP-PICK1 at 70 sec.

inhibitor does not alter the translocation [29]. As shown in Figure 2, the pretreatment with a PKC inhibitor did not inhibit the translocation of α PKC-GFP, but simultaneous translocation of PICK1 was completely blocked by 7 nM Gö6983, a PKC inhibitor. These findings show that TPA-induced translocation of PICK1 requires the activation of α PKC in addition to the overexpression of α PKC. Why is the activation of α PKC necessary? One possibility is that the phosphorylation of PICK1 by PKC is required for the translocation of PICK1 accompanied with α PKC. The other possibility is that the QSAV sequence at the extreme COOH-terminus of α PKC may become accessible to the PDZ binding domain of PICK1 once α PKC is activated and has an open conformation. As PICK1 has three potential PKC-phosphorylation sites, we mutated the phosphorylation sites (T82, Y227 and T249) to alanine residues and examined the TPA-induced translocation of the PICK1 mutants. The

mutation of T82 but not that of T227 or T249 abolished the translocation of PICK1, suggesting that the phosphorylation of T82 was important. However, as T82 is localized in the PDZ binding domain of PICK1, the mutation of T82 may inhibit the translocation of PICK1 by disturbing the interaction between the PDZ binding domain of PICK1 and α PKC but not by the inhibition of the phosphorylation-dependent process. If the phosphorylation of T82 is responsible for the translocation of PICK1, PICK1-T82E, which mimics the phosphorylated form of PICK1, could be translocated by TPA. Substitution of T82 to glutamate in PICK1 also abolished the TPA-induced translocation of PICK1 accompanied with α PKC (data not shown). These results suggested that T82 might be important for the interaction between PICK1 and α PKC, although the necessity of T82 phosphorylation remains to be controversial.

PDZ domain proteins can organize signaling com-

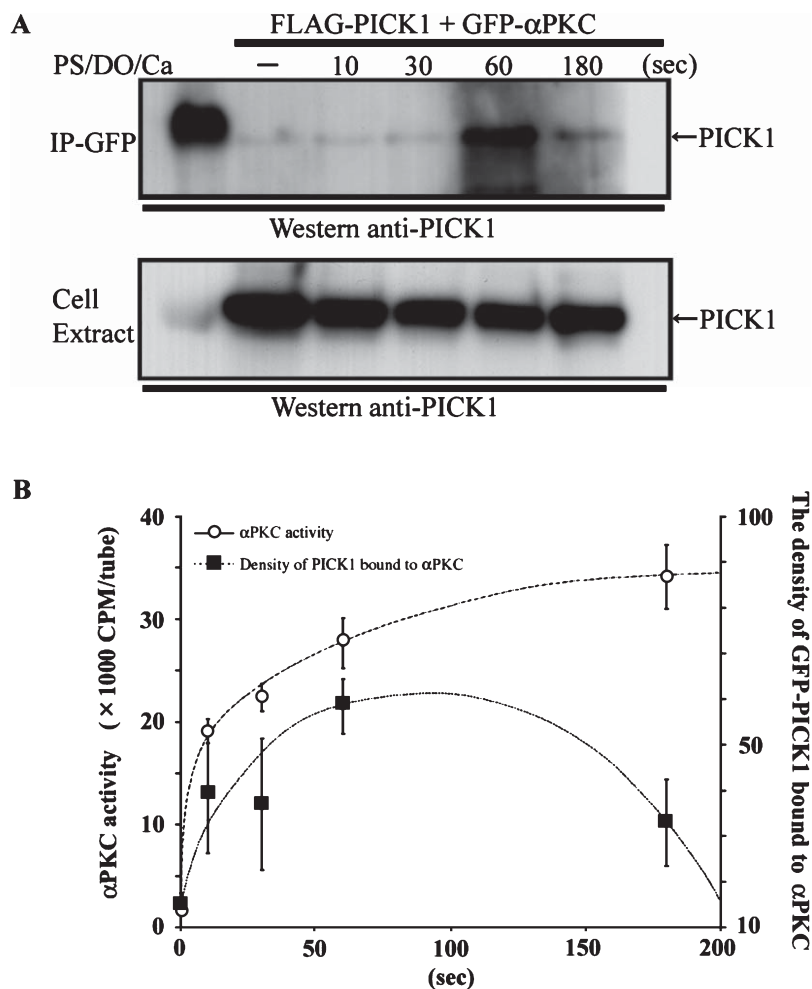


Fig. 4. Time course of PKC activity and interaction between PICK1 and α PKC after PKC activation. **A:** FLAG-PICK1 and GFP- α PKC were co-expressed in HEK293 cells and the homogenate of the HEK293 cells was treated with PS/DO/Ca²⁺. The complex of PICK1 and α PKC was immunoprecipitated with anti-GFP antibody at each time point after the treatment with PS/DO/Ca²⁺. The amount of complex was analyzed by western blotting using anti-PICK1 antibody (upper row). The expression of FLAG-PICK1 was confirmed by western blot analysis of the cell extracts using anti FLAG antibody (lower row). **B:** For kinase assay, α PKC was immunoprecipitated with anti GFP antibody at each time point after the treatment with PS/DO/Ca²⁺. The kinase activity of α PKC was assayed as described in Materials and Methods. All results represent the means and standard errors of more than three determinations. The data obtained are summarized in **B**.

plexes and regulate channel activity, trafficking, and localization [2, 11, 12, 17, 30]. PICK1 was first identified as a binding partner of α PKC [33, 34] and has since been shown to interact with many receptors, channels, and transporters [3, 4, 7–9, 14, 35, 37, 41]. Although PICK1 has only one PDZ domain, PICK1 oligomerization through its coiled-coil region [34, 41] enables it to cross-link with different binding partners. PICK1 interacts with the C terminal of AMPA receptors [6, 41] and has been implicated in the internalization or stabilization of internal pools of AMPA receptors during cerebellar and hippocampal long term depression [13, 16, 28, 42]. Perez *et al.* [28] suggested that PICK1 functions as a targeting and transport protein that directs the activated form of α PKC to GluR2 in spines, leading to the activity-dependent release of GluR2 from synaptic anchor proteins and the PICK1-dependent transport of GluR2 from

the synaptic membrane [28]. The present study, however, demonstrated that α PKC targeted PICK1 to the membrane, and that this transport occurred through the phosphorylation of PICK1. The binding of PICK1 and α PKC appeared to be transient, since PICK1 was separated from α PKC after the phosphorylation by α PKC.

Real time imaging can reveal when and where α PKC binds to PICK1. However, as TPA induced the slow and simultaneous translocation of α PKC and PICK1 from cytosol to membrane, it was difficult to determine whether the binding of α PKC with PICK1 precedes their translocation to the plasma membrane, or whether the translocation precedes their binding. Therefore, we examined the temporal difference between the translocations of α PKC and PICK1 after the stimulation with UTP which induced the transient membrane translocation of α PKC (Fig. 3). UTP translocated

α PKC earlier than PICK1 and subsequently α PKC was released from the plasma membrane earlier than PICK1, suggesting that UTP induces the activation and translocation of α PKC, the binding of the activated α PKC with PICK1, the phosphorylation of PICK1, and finally the dissociation of PICK1 from α PKC. Although the activation of α PKC is necessary for PICK1 to translocate to the plasma membrane as shown in Figure 2, the binding of α PKC to PICK1 did not continue during the localization of PICK1 on the plasma membrane. Figure 4 revealed that once PICK1 was translocated to the plasma membrane, its interaction with α PKC was no longer necessary for the membrane localization of PICK1. It is possible that the phosphorylation of PICK1 by α PKC is responsible for the association of PICK1 with unknown proteins on the plasma membrane such as receptors. But once PICK1 is anchored on the membrane, the binding to α PKC is no longer necessary for PICK1 and the unbound PICK1 can then regulate the receptors/transporters on the membrane.

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