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 Cα (αPKC) binding protein using the yeast two-hybrid system. In addition to αPKC, 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 αPKC. We examined the spatiotemporal interaction of PICK1 and PKC using live imaging techniques and showed that the activated αPKC binds to PICK1 and transports it to the plasma membrane. Although the membrane translocation of PICK1 requires the activation of αPKC, PICK1 is retained on the membrane even after PKC moves back to the cytosol. These results suggest that the interaction between αPKC and PICK1 is transient and may not be necessary for the regulation of receptors/transporters by PICK1 or by αPKC 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 (αPKC; α, βI, βII, 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-methylisoxazole-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 αPKC through interaction with the QSAV sequence at the extreme COOH-terminus of αPKC [34]. The carboxylate-binding
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], mGlur7α receptor [3, 7], and dopamine and norepinephrine transporters [38]. PICK1 forms a triple complex containing αPKC and mGlur7 in neurons [7].

In the present study, we examined the spatio-temporal interaction between PICK1 and αPKC 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 and 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 Regent (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-αPKC fusion protein in which αPKC (BS611) and DsRed2 were bound at the N-terminus of αPKC. BS1085 was a plasmid encoding DsRed2-γPKC fusion protein in which γPKC in pTB701 (BS55) and DsRed2 were bound at the N-terminus of γPKC. BS1104 was a plasmid encoding DsRed2-γPKC-CTα fusion protein. A cDNA fragment of 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 γPKC in pTB701 (BS55) as the template. The sense and antisense primers were 5'-TTAAGC TTCCATGGCG-

GGTCTGGTCCTG-3' and 5'-TTGAATTCTCATACTGGCCTTTGCA AGATTGGGTGCACGAAGTCCGG GTT-CA-3', respectively. The PCR product of γPKC 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 HeNe1 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-αPKC 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-αPKC

GFP-αPKC was immunoprecipitated from HEK293 cells transfected with GFP-αPKC 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-32P]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 µm² 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-αPKC was observed in the perikarya of the transfected HEK293 cells which express DsRed2-αPKC 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-αPKC 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-αPKC 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-αPKC 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-αPKC translocated from the cytosol to the plasma membrane within 20 min after the treatment with TPA. GFP-PICK1 fusion protein was not translocated by TPA when co-expressed with βIPKC or γPKC.
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 αPKC.

Staudinger et al. reported that PICK1 interacts with the PDZ binding site in the C terminus of αPKC 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 γPKC or βIPKC. As shown in Figure 1C and 1D, TPA failed to translocate GFP-PICK1 to the plasma membrane, while the TPA-induced translocation of γPKC or βIPKC was evident, suggesting that the translocation of

![Fig. 2. A: Role of C-terminal region of αPKC for TPA-induced translocation of PICK1 in HEK293 cells. Activation of PKC by 1 μM TPA induced the obvious translocation of GFP-PICK1 and DsRed2-γPKC which had the C-terminal region of αPKC (DsRed2-γPKC-CTα) from the cytosol to the plasma membrane. B: Effects of αPKC activity on PICK1 translocation. Pretreatment with 7 nM Gö6983 (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 αPKC. 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 αPKC. 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 αPKC.](image_url)
PICK1 is related with the expression of αPKC but not γPKC or βPKC. As it was supposed that αPKC binds to PICK1 through its C terminal with PDZ domain of PICK1, the binding domain of αPKC to translocate with PICK1 was further examined by using γPKC having C terminal of αPKC. The four amino acid residues at the C terminal of αPKC have been shown as a PDZ-binding site. The last four amino acids of γPKC were substituted with PDZ-binding sequence of αPKC (γPKC-αCT) and co-expressed with PICK1. As shown in Figure 2A, the simultaneous translocation of GFP-PICK1 with γPKC-αCT to the plasma membrane was observed after TPA treatment.

To study whether not only the binding of αPKC to PICK1 but also the phosphorylation of PICK1 by αPKC 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-αPKC (Fig. 2B). In the presence of 7 nM Gö6983, neither the cytosolic localization of PICK1 nor that of αPKC was altered. Gö6983, however, blocked the translocation of GFP-PICK1, while DsRed2-αPKC showed membrane translocation. The results indicate that the TPA-induced translocation of PICK1 requires both the αPKC activation in addition to the binding of αPKC 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 αPKC after its binding to active αPKC 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 were 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 αPKC was evident after TPA treatment in all HEK293 cells expressing these PICK1 mutants. These results suggested that the TPA- and αPKC-dependent translocation of PICK1 to the plasma membrane requires the phosphorylation of threonine 82 (T82).

As shown above, TPA-induced translocation of αPKC was slow and irreversible. Therefore, it is difficult to detect the temporal difference of the translocation between αPKC and PICK1. We used UTP stimulation to translocate αPKC, which induces a rapid and reversible translocation [32]. Activation of αPKC through G protein-coupled receptor stimulation induced rapid and reversible translocation of both DsRed2-αPKC and GFP-PICK1 but the time course of the membrane targeting differed between DsRed2-αPKC and GFP-PICK1 (Fig. 3). The temporal difference of the two translocations is shown in Figure 3A. The fluorescence of DsRed2-αPKC 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 µM 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 αPKC precedes that of PICK1, and that PICK1 is retained on the membrane longer than αPKC. These findings suggested that once PICK1 is targeted to the plasma membrane by the association with the activated αPKC, it is not necessary for PICK1 to associate with αPKC.

The relation between the kinase activity of αPKC and its binding to PICK1 was further analyzed (Fig. 4). The time course of PKC activity was assayed by measuring the incorporation of 32Pi from [γ-32P]ATP into MBP at each time point after the addition of the activators, PS/DO/Ca2+ as described in Materials and Methods. αPKC was significantly activated 10 sec after the treatment with PS/DO/Ca2+ and the kinase activity of αPKC was progressively increased at least until 180 sec. However, pulldown assay using anti-GFP antibody showed that the maximal interaction of PICK1 with GFP-αPKC was found 60 sec after the activation of GFP-αPKC, 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 αPKC continuously even when αPKC 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 αPKC. PICK1 translocated to the plasma membrane within 30 min in response to TPA as αPKC was translocated. This PKC-dependent translocation of PICK1 was αPKC-specific, although other PKC subtypes such as βPKC and γPKC showed TPA-induced translocation similar to that of αPKC. It is suggested that the subtype specific interaction of αPKC with PICK1 is caused by the binding of PDZ domain of PICK1 to the PDZ-binding site (QSAV) at the COOH terminus of αPKC [34]. Indeed, TPA-induced translocation of PICK1 to the plasma membrane was observed when γPKC which has a QSAV sequence at C-terminal was co-expressed, indicating that the PDZ-binding sites of αPKC, QSAV, is necessary for PKC-dependent translocation of PICK1. However, TPA failed to translocate PICK1 without overexpression of αPKC, although αPKC is expressed endogenously in HEK293 cells. Significant amounts of αPKC 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...
inhibitor does not alter the translocation [29]. As shown in Figure 2, the pretreatment with a PKC inhibitor did not inhibit the translocation of αPKC-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 αPKC in addition to the overexpression of αPKC. Why is the activation of αPKC necessary? One possibility is that the phosphorylation of PICK1 by PKC is required for the translocation of PICK1 accompanied with αPKC. The other possibility is that the QSAV sequence at the extreme COOH-terminus of αPKC may become accessible to the PDZ binding domain of PICK1 once αPKC 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 αPKC 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 αPKC (data not shown). These results suggested that T82 might be important for the interaction between PICK1 and αPKC, although the necessity of T82 phosphorylation remains to be controversial.

PDZ domain proteins can organize signaling com-

Fig. 3. A: Simultaneous observation of UTP-induced translocation of GFP-PICK1 and DsRed2-αPKC. Change in the fluorescence of GFP-PICK1 and DsRed2-αPKC expressed in CHO-K1 cells by 100 µM UTP at 37°C. Activation of PKC by 100 µM UTP induced the obvious translocation of DsRed2-αPKC 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-αPKC after UTP stimulation. Membrane/cytosol ratio of DsRed2-αPKC peaks at 30 sec, while that of GFP-PICK1 at 70 sec.
plexes and regulate channel activity, trafficking, and localization [2, 11, 12, 17, 30]. PICK1 was first identified as a binding partner of αPKC [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 αPKC 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 αPKC targeted PICK1 to the membrane, and that this transport occurred through the phosphorylation of PICK1. The binding of PICK1 and αPKC appeared to be transient, since PICK1 was separated from αPKC after the phosphorylation by αPKC.

Real time imaging can reveal when and where αPKC binds to PICK1. However, as TPA induced the slow and simultaneous translocation of αPKC and PICK1 from cytosol to membrane, it was difficult to determine whether the binding of αPKC 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 αPKC and PICK1 after the stimulation with UTP which induced the transient membrane translocation of αPKC (Fig. 3). UTP translocated

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**Fig. 4.** Time course of PKC activity and interaction between PICK1 and αPKC after PKC activation. **A:** FLAG-PICK1 and GFP-αPKC were co-expressed in HEK293 cells and the homogenate of the HEK293 cells was treated with PS/DO/Ca²⁺. The complex of PICK1 and αPKC 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, αPKC was immunoprecipitated with anti GFP antibody at each time point after the treatment with PS/DO/Ca²⁺. The kinase activity of αPKC 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**.
αPKC earlier than PICK1 and subsequently αPKC was released from the plasma membrane earlier than PICK1, suggesting that UTP induces the activation and translocation of αPKC, the binding of the activated αPKC with PICK1, the phosphorylation of PICK1, and finally the dissociation of PICK1 from αPKC. Although the activation of αPKC is necessary for PICK1 to translocate to the plasma membrane as shown in Figure 2, the binding of αPKC 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 αPKC was no longer necessary for the membrane localization of PICK1. It is possible that the phosphorylation of PICK1 by αPKC 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 αPKC is no longer necessary for PICK1 and the unbound PICK1 can then regulate the receptors/transporters on the membrane.

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