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Serine 77 in the PDZ domain of PICK1 is a protein kinase C α phosphorylation site regulated by lipid membrane binding

Ina Ammendrup-Johnsen, Thor S. Thorsen, Ulrik Gether, and Kenneth L. Madsen*

Molecular Neuropharmacology Laboratory, Lundbeck Foundation Center for Biomembranes in Nanomedicine, Department of Neuroscience and Pharmacology, Panum Institute, University of Copenhagen, 2200 Copenhagen N, Denmark

Abstract

PICK1 (protein interacting with C-kinase 1) contains an N-terminal protein binding PDZ domain and a C-terminal lipid binding BAR domain. PICK1 plays a key role in several physiological processes including synaptic plasticity. However, little is known about cellular mechanisms governing the activity of PICK1 itself. Here we show that PICK1 is a substrate *in vitro* both for PKC α (Protein kinase C α), as previously shown, and for CaMKII α (Ca²⁺-calmodulin dependent protein kinase II α). By mutation of predicted phosphorylation sites, we identify Ser77 in the PDZ domain as a major phosphorylation site for PKC α . Mutation of Ser77 reduced PKC α mediated phosphorylation ~50% whereas no reduction was observed upon mutation of seven other predicted sites. Addition of lipid vesicles increased phosphorylation of Ser77 ~10-fold, indicating that lipid binding is critical for optimal phosphorylation. Binding of PKC α to the PICK1 PDZ domain was not required for phosphorylation but a PDZ domain peptide ligand reduced overall phosphorylation ~30%. The phosphomimic S77D reduced cytosolic clustering of eYFP-PICK1 in COS7 cells and thereby conceivably its lipid binding and/or polymerization capacity. We propose that PICK1 is phosphorylated at Ser77 by PKC α preferentially when bound to membrane vesicles and that this phosphorylation in turn modulates its cellular distribution.

Keywords

PICK1; PDZ domain; phosphorylation; BAR domain proteins; lipidbinding

PICK1 (Protein Interacting with C Kinase 1) is a scaffolding protein first identified as an interaction partner for PKC α (protein kinase C α) (1). Functional studies have suggested that PICK1 plays a key role in molecular mechanisms underlying synaptic plasticity (2). Furthermore, PICK1 has been implicated in diverse pathophysiological processes such as schizophrenia (3, 4), neuropathic pain (5, 6), cocaine addiction (7), addiction relapse (8), cerebral ischemia (9, 10), breast cancer (11) and defects in spermatogenesis (12, 13).

PICK1 contains an N-terminal protein binding PDZ (PSD-95/Discs-large/ZO-1) domain and a C-terminal lipid binding BAR (Bin/amphiphysin/Rvs) domain (14). The PDZ domain of PICK1 has been reported to interact with more than 40 proteins in addition to PKC α (15, 16). Most of the known ligands are the C-termini of different membrane proteins such as the

*Corresponding author: Department of Neuroscience and Pharmacology, Panum Institute 18.6, University of Copenhagen, DK-2200 Copenhagen N, Denmark. Phone +45 355327549, Fax +45 35327610, KENNETHMA@sund.ku.dk .

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GluA2/A3 subunits of the AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid)-type ionotropic glutamate receptor (17), the metabotropic glutamate receptor mGluR7 (18), kainate-type ionotropic glutamate receptors (19), ASIC (Acidic Sensing Ion Channel) I and II (20) as well as various neurotransmitter transporters including the dopamine transporter (DAT) (21, 22) and the GLT1b glutamate transporter (23).

BAR domains represent dimeric α -helical modules that possess a distinctive capability to both sense and impose membrane curvature (24-26). Thus, PICK1 is likely to exert its function in relation to its several PDZ binding partners via curvature dependent lipid membrane interactions. Interestingly, the PDZ domain itself has been implicated in mediating lipid membrane binding (27). There is also strong evidence that the lipid binding capacity of the PICK1 BAR domain is autoinhibited and that release of this inhibition requires ligand binding to the PDZ domain in conjunction with membrane recruitment of PICK1 (28, 29). The PICK1 BAR domain mediates moreover dimerization of PICK1 and in doing so it enables the protein to bring two PDZ domain binding partners in close proximity (16),

A growing number of studies have provided increasing insight into the cellular mechanisms underlying the various functions of PICK1. In synaptic plasticity, PICK1 is believed to mediate a decrease of the surface expression of postsynaptic GluA2 containing AMPA receptors, a key process in LTD (long term depression) (30, 31). PICK1 is also suggested to mediate the presynaptic clustering of mGluR7 (32) by facilitating phosphorylation and surface stabilization of mGluR7 by PKC α (33). In relation to ASICs, PICK1 seems critical for the PKC α mediated regulation of ASIC currents and plasmamembrane expression (34, 35). ASICs are both involved in the processes underlying hippocampal synaptic plasticity (36) and in patophysiological processes such as hyperalgesia (37). PICK1 has furthermore been suggested to regulate surface expression of other interaction partners such as GLT1b and DAT (21, 23).

Despite the involvement of PICK1 in a multitude of different cellular functions, little is known about regulatory mechanisms controlling PICK1 activity. PICK1 has been shown to be a substrate for PKC α (1) and suggested to become phosphorylated following activation of Ca²⁺/calmodulin-dependent kinase II α (CaMKII α) although direct phosphorylation was not shown (38). Here we show that indeed PICK1 is a direct substrate for PKC α as well as for CaMKII α . By applying a systematic site directed mutagenesis strategy, we map Ser77 in the PDZ domain of PICK1 as the major *in vitro* PKC α site. We show that the presence of lipid vesicles dramatically increases PKC α mediated PICK1 phosphorylation whereas binding of peptide ligand to the PDZ domain decreases it. Finally, we find that mimicking the phosphorylated state of Ser77 by mutation of the residue to aspartate (S77D) markedly reduces spontaneous clustering of eYFP (enhanced Yellow Fluorescent Protein)-PICK1 in transfected COS7 cells, consistent with possible impairment of lipid binding and/or polymerization. Taken together, the data identifies a PKC α phosphorylation site in PICK1 that affects the cellular distribution of the PICK1 molecule and thus its regulation of PDZ binding partner function.

MATERIALS AND METHODS

Molecular biology

The Quick Change® method (Stratagene, La Jolla, CA, USA) was used for single amino acid substitutions. As template we used the pET41 plasmid (Stratagene) encoding GST (glutathione-S-transferase) coupled rat PICK1 (GST-PICK1) as previously described (39) or the peYFP-C1 plasmid (Clontech) encoding eYFP-PICK1 as described (28). GST-PICK1 (136-416) was generated similar to Gst-PICK1 by amplification of residue 136-416 of rat

PICK1 from a pcNEO vector (Kind gift from Dr Kumlesh Dev). The primers introduced an N-terminal MunI and a C-terminal AvrII site which were used to clone the fragment into the pET41 vector. All primers were obtained from DNA technology A/S, Aarhus, Denmark. All mutant sequences were verified by restriction enzyme mapping and DNA sequencing and transformed into BL21 DE3 pLysS (Novagen).

Protein purification

GST-PICK1 wild type (WT) or mutants and GST-PICK1 (136-416) were purified as described previously (39), briefly, 50 mL LB media containing 34 µg/mL chloramphenicol (MP Biomedicals) and 50 µg/mL kanamycin (Invitrogen) was inoculated with *E. Coli* BL21 DE3 pLys S containing the pET41 vector encoding the appropriate PICK1 sequence. The bacteria were grown overnight at 37°C, then diluted into 1 L of LB media containing 34 µg/mL chloramphenicol and 50 µg/mL kanamycin, and grown at 30°C. When the right OD (0.6-1.0 at 600 nm) was obtained, protein expression was induced by adding Isopropyl β-D-Thiogalactopyranoside (Sigma-Aldrich) to a final concentration of 0.5 mM. After 3 hours of induction at 30 °C, the bacteria were pelleted at 8000 × g for 12 minutes. The pellets were resuspended on ice in 25 mL ice cold lysis buffer (50 mM Tris base pH 7.4 (Angus Chemicals), 125 mM NaCl (Merck), 1% Triton X-100 (Sigma-Aldrich), 20 µg/mL DNase 1 (Sigma-Aldrich) and 1 mM dithiothreitol (DTT) (Sigma-Aldrich)). The lysates were frozen for 40 minutes at –80°C, and thawed again in a 4 °C waterbath, in order to achieve proper lysis of the bacteria. The cell debris were subsequently pelleted at 47,000 × g for 30 minutes, and the supernatant transferred to new tubes. For each batch, 0.75 mL glutathione sepharose™ 4B beads (Amersham Bioscience) were washed 3 times in washing buffer (50 mM Tris, 125 mM NaCl, 0.01% Triton X-100 and 1mM DTT) and then added to the supernatant. GST coupled proteins were allowed to bind to the glutathione beads by incubation for 90 minutes at 4 °C under continuous rotation. Next, the beads were pelleted at 3000 × g for 5 minutes and washed three times with washing buffer. Triton X-100 0.1 % was used in wash buffer instead of 0.01 % for proteins purified for the fluorescence polarization assay. In order to cleave the protein from the GST domain and the coupled beads, the beads were resuspended in 0.5 mL washing buffer containing 0.3 U Thrombin (Novagen), transferred to Bio-Spin® disposable chromatography columns (Bio-Rad) and incubated overnight at 4 °C with rotation.

In vitro phosphorylation - PKCα

For each reaction, approximately 3 µg of purified protein was diluted in protein washing buffer to a final volume of 10 µL, and then mixed with 10 µL reaction buffer (120 mM Tris pH 7.5, 60 mM MgCl₂ (Merck), 6 mM CaCl₂ (Merck)), 5 µL PKC Lipid Activator (Upstate, Frederikssund, Denmark) and 10 ng PKCα (Upstate). Finally, 2 µCi γ-³²P ATP (Amersham Bioscience, Pb108) was added and the mixture incubated at 30 °C for 30 minutes. The reaction was stopped by placing the mixture on ice and adding 10 µL of SDS-PAGE loading buffer (200 mM Tris-HCl, pH=6.8, 400 mM DTT, 8 % SDS, 0.4 % Bromophenol blue, 40 % Glycerol). The samples were analyzed by 10% SDS-PAGE. The front of the gel, which contained all unbound ATP, was subsequently removed, and the gel was washed 3 times 10 minutes in distilled water before staining with GelCode® Blue Stain Reagent (Thermo Scientific). The gel was finally wrapped in plastic, placed in a cassette with a storage phosphor screen (Amersham Bioscience) and left overnight. The screen was scanned with a Molecular Imager FX (BioRad). The coomassie stained gel was scanned with a Fluor-S™ MultiImager (BioRad). The densities of the protein bands as well as the phosphorylation bands were determined by use of the Discovery Series Quantity One 1-D Analysis Software Version 4.5 (BioRad). The ratio between the phosphorylation density and the protein density was calculated and compared to the ratio determined for PICK1 WT which was defined to be 100%.

In vitro phosphorylation – CaMKII α

For each reaction, approximately 3 μ g of purified protein was diluted in protein washing buffer to a final volume of 15 μ L. The reaction buffer (100 mM Hepes (Sigma-Aldrich), 20 mM MgCl₂, 1 mM CaCl₂, 2 mM DTT, 200 μ g/mL BSA (Sigma-Aldrich), 0.6 mM ATP (Sigma-Aldrich), 2 μ M Calmodulin (Sigma-Aldrich), 30 nM CaMKII α (kindly provided by R.J. Colbran)) was mixed and incubated for 10 minutes at 30 °C. 15 μ L of reaction buffer was then mixed with 15 μ L of protein before adding 2 μ Ci γ -³²P ATP and incubation at 30 °C for 10 minutes. The reaction was stopped by placing the mixture on ice and adding 10 μ L of SDS-PAGE loading buffer. The SDS-PAGE procedure was the same as described for the PKC α assay. The results were calculated as explained for PKC α assay, except that the density of the CaMKII α auto-phosphorylation band was subtracted from the density of the PICK1 phosphorylation.

Preincubation with vesicles or peptides prior to phosphorylation by PKC α

To investigate the effect of vesicle binding on PKC α mediated phosphorylation of PICK1, the protein was preincubated in 10 μ L protein washing buffer with 3 μ g of vesicles prepared from Folch fraction type I (SigmaAldrich B1502) and extruded to obtain a diameter size of 100 nm. The mix was preincubated for 30 minutes on ice, control samples without vesicles where treated the same way. To investigate the effect of peptide binding, either DAT C13 or DAT C13 V to D (see fluorescence polarization section for a description of peptides) was added to each sample to a final concentration of 67 μ M, and incubated 10 minutes at 30°C prior to addition of reaction buffer, kinase activator, PKC α and 2 μ Ci γ -³²P ATP (Amersham Bioscience, Pb108).

Fluorescence Polarization assay

All peptides were purchased from Schafer-N (Copenhagen, Denmark). The peptides corresponded to the 13 C-terminal residues of the human DAT, PKC α and GluA2, respectively. In addition to the DAT WT peptide (DAT C13), a mutant with the C-terminal Val substituted with Asp was used (DAT C13 V to D). DAT C13: EVRQFTLRHWLKV; DAT C13 V to D: EVRQFTLRHWLKD; PKC α C13: NPQFVHPILQSAV; GluA2 C13: EGYNVYGIESVKI. Fluorescence polarization binding experiments were carried out as described in (39). Briefly, saturation experiments were performed with DAT C13 with an additional N-terminal cysteine residue coupled to the fluorophore Oregon Green 488 (Schafer-N), referred to as OrG DAT C13. The assay was carried out in 96 wells microtiter plates (black, 1/2 area, non-binding-surface, Corning). Purified protein was diluted in 100 μ L of TBS (50 mM Tris, pH 7.4, 125 mM NaCl) resulting in a 1/3 log dilution row with 12 points spanning 4 decades for each protein. OrG DAT C13 was added to each well to a final concentration of 6.7 nM, the plate was incubated on ice for 10 minutes before reading in a Chameleon Fluorescence Polarization Reader (Hidex) in the FP mode, using a 488 nm excitation filter and a 535 nm long pass emission filter. FP was calculated according to the equation $FP = (I_v - g \cdot I_h) / (I_v + g \cdot I_h)$, I_v is the intensity in the vertical plane and I_h the intensity in the horizontal plane, the apparent K_d was determined by the equation

$$Y = \left(FP_{\text{boundligand}} - FP_{\text{freeligand}} \right) \cdot [PICK1] / (K_d + [PICK1]) + FP_{\text{freeligand}}$$

The concentration of the different PICK1 variants was calculated based on the apparent K_d obtained from the saturation binding experiments so equal amounts of peptide binding proteins were used in each experiment. The competition assay was carried out using 96-well microtiter plates in which 12 wells were used for each binding curve. 45 μ L of protein in an appropriate dilution were added to a 1/3 log dilution row of peptide diluted in 45 μ L of TBS.

Finally 10 μ L of 0.067 μ M DAT OrG was added, the plate was incubated on ice for 10 minutes before it was read. The obtained fluorescence polarization values were analyzed by non-linear regression analysis in Prism 5.0 (GraphPad Software Inc., San Diego, CA) and the affinities for the peptides were calculated as described (39).

Vesicle pull-down assay

The lipid film was prepared as described previously (40) from Folch fraction type I and rehydrated in 300 mM D-sorbitol to final concentration of 50 mg/ml. The samples were sonicated, vortexed and extruded 15 times through a filter with pore size of 100 nm. For pull-down, 10 μ M protein was incubated with 10 mg/ml vesicles in 150 μ L TBS (Tris-Buffered Saline pH 7.4) containing 1mM DTT for 30 minutes on ice and spun down at 140.000xg for 15 minutes at 4°C. The supernatant was transferred to new tubes and the pellet was resuspended in 150 μ L TBS with 1 mM DTT, and supernatant and pellet were analysed by 10% SDS PAGE, stained with GelCode® Blue Stain Reagent (Thermo Scientific) and relative protein levels were quantified with AlphaEaseFC software (Alpha Innotech Corporation).

Cell culture and transfection

COS7 cells were grown in DMEM (Dulbecco's modified Eagle's medium) 1965 with 10% foetal calf serum (HyClone, Logan, USA), 2 mmol/L glutamine and 0.01 mg/mL gentamicine (Invitrogen) at 37°C in a humidified 5% CO₂ atmosphere. The transfections were performed as reverse transfection, i.e. a mix of 0.1 μ g C1-eYFP-PICK1 plasmid, 0.3 μ L lipo2000 (Invitrogen) and 47 μ L OptiMEM (Invitrogen) was spotted in the well of an 8-well Lab-Tek chambered coverglass (#155411, Nunc), incubated for 20 minutes before addition of 40.000 cells in 50 μ L OptiMEM. After 5 hours, the media was changed to normal growth media. The cellular distribution of eYFP-PICK1 was analyzed 24-48 hours after transfection in live cells by epifluorescence microscopy (Carl Zeiss TM210). Images were acquired with a 40X oil objective and a YFP dichroic filter (excitation at 514 nm). Image acquisition was performed with MetaMorph (Meta Imaging, Universal Imaging, V.4.6.), settings were fixed throughout an experiment. Images were analysed with the ImageJ software. The counting of cells containing cluster was performed manually. A cluster was defined as an area with an intensity of 1.5 times higher than the average intensity in the cytosol. At least 10 pictures were taken and approximately 50 cells were counted of each sample.

Western blotting

COS7 cells (2×10^6) transfected with C1-eYFP-PICK1 plasmid were lysed in 1 mL TrisHCl, pH 7.4 containing 1 % (v/v) Triton X-100, 5 mM n-ethyl-maleimide and protease inhibitor cocktail (Roche Diagnostics). After solubilization for 30 minutes at 4°C, cell was removed by centrifugation at 16000 x g for 20 minutes at 4°C. Protein concentration was determined in supernatant and equal protein amounts of each sample were analyzed by 10% SDS-PAGE as described above. After blotting to PVDF membrane, the membrane was split in to two around 50 kDa and eYFP-PICK1 was detected in the top part with by 1:1000 rabbit anti GFP (Abcam ab 290) and actin was detected in the lower part by 1:2000 rabbit anti actin (Sigma A2066), and then 1:5000 secondary HRP-conjugated antibodies (Pierce). The signal was quantified using the ECL kit (Amersham) and FluorChem HD2 (Alpha Innotech Corporation) and the AlphaEaseFC software (Alpha Innotech Corporation). The level of eYFP-PICK1 was normalized to the corresponding actin level, and then the level of eYFP-PICK1 S77D was calculated relative to eYFP-PICK1 WT.

RESULTS

PICK1 is an *in vitro* substrate for PKC α and CaMKII α

In vitro phosphorylation experiments with purified PICK1 confirmed that PICK1 is a substrate for PKC α (Fig. 1 A) as previously described (1). SDS-PAGE of the phosphorylation sample revealed a [32 P] labelled band eluting corresponding to the elution of purified PICK1 (MW ~50 kDa) visualized by a BlueGel stain (Fig. 1A). *In vitro* phosphorylation experiments also indicated that PICK1 is a substrate for CaMKII α at least *in vitro* (Fig. 1B and C). CaMKII α is autophosphorylated and because of the similar molecular sizes of CaMKII α and PICK1 it was not possible to separate the two proteins by SDS-PAGE. Therefore, CaMKII α was preincubated with unlabelled ATP prior to phosphorylation of PICK1 to minimize the background signal from CaMKII α autophosphorylation (Fig. 1B). In order to conclusively separate the signal from PICK1 and CaMKII α phosphorylation, the experiment was also performed with N-terminally truncated PICK1 (136-416), which would be separated from CaMKII α by SDS PAGE (Fig. 1C). Fig. 1B and C demonstrate together that PICK1 is a substrate for CaMKII α *in vitro*, and that phosphorylation, at least to some extent, takes place beyond residue 135.

Identification of a PKC α phosphorylation site in PICK1

To map the PKC α phosphorylation site(s) in PICK1 we first identified putative sites by use of the NetPhos prediction program (41). We chose to analyse the eight serine or threonine residues with the highest predicted phosphorylation probability score for being a phosphorylation site (>0.85). The probability scores are presented in Fig. 2A together with the position of the potential phosphorylation sites in a schematic figure of PICK1. All eight residues were substituted with aspartate one at a time and the resulting mutants were purified upon expression in *E. coli*. The purified mutants were analyzed by *in vitro* phosphorylation and the phosphorylation levels quantified by phosphoimaging analysis. Fig. 2B shows the quantifications of the relative phosphorylation level of PICK1 mutants by PKC α for 3-5 experiments. Substitution of Ser77 with aspartate significantly reduced the phosphorylation compared to PICK1 WT ($39\% \pm 7.7\%$, $p < 0.01$, $n=4$). A similar reduction was seen by mutating Ser77 to alanine ($47\% \pm 7.7\%$, $p < 0.05$, $n=3$) (Fig. 2B). None of the other substitutions resulted in phosphorylation significantly different from WT. This strongly suggests that Ser77 in the PICK1 PDZ domain is a PKC α phosphorylation site. Moreover, the extent of the decrease suggests that Ser77 is the most prominent PKC α site at least *in vitro*.

We attempted to validate that Ser77 represents a PKC α phosphorylation site also in a cellular context according to Olsen et al. (Olsen et al, 2006) by use of mass spectrometry analysis after isolation of PICK1 tagged at the N-terminus with enhanced yellow fluorescent protein (eYFP) from transfected HEK293 cells. However, due to an unusually high lysine/arginine content in the area around Ser77 in the primary structure of PICK1, we were unable to isolate a peptide containing Ser77 and thus unable to determine its cellular phosphorylation state (data not shown).

The Ser/Thr mutants described above, as well as Thr289 substituted with aspartate, were also tested in the CaMKII α *in vitro* phosphorylation assay. T289 was not predicted by Netphos but is positioned in a CaMKII α consensus site (42). However, none of the substitutions reduced CaMKII α *in vitro* phosphorylation (Fig. 2C).

Vesicle binding increases phosphorylation of PICK1 by PKC α PICK1 can bind to lipid membranes both via its BAR domain (14) and via a putative lipid binding patch of the PDZ domain (27). Because PKC α also is capable of binding to lipid membranes (43) we envisioned that the presence of a lipid membrane could act as a scaffold to facilitate

phosphorylation by bringing both PKC α and PICK1 to the membrane. Furthermore, it is known that the affinity of some PKC substrates can be enhanced by lipid induced α -helix formation (44). Conversely, Ser77 is located close to the reported lipid binding patch (the CPC motif) and could be shielded by lipid membrane binding (Fig. 3A). We decided, therefore, to test whether the presence of lipid vesicles would affect phosphorylation of PICK1 by PKC α . Addition of vesicles (prepared from total brain lipids and extruded to obtain a maximal diameter size of 100 nm) dramatically increased the phosphorylation of PICK1 by PKC α ($971\% \pm 197\%$, $P < 0.01$, $n=3$) (Fig. 3 B). Moreover, this effect was almost abolished by in the phosphomimic PICK1 S77D. This reduction could result from either decreased vesicle binding by PICK1 due to introduction of a negative charge close to the positively charged lipid binding patch or direct removal of the phosphorylation site sensitive to vesicle binding. To discriminate between these possibilities we also tested the S77A substitution to mimic the unphosphorylated state of Ser77. Introduction of S77A likewise reduced phosphorylation making it unlikely that the observed difference between PICK WT and S77 substitution mutants is caused by decreased vesicle binding of the mutants. Therefore, we conclude that incubation with vesicles increases *in vitro* phosphorylation of PICK1 by PKC α and that this effect is predominantly due to an increased phosphorylation of Ser77.

Substitution of Ser77 does not affect the peptide binding capacity of the PICK1 PDZ domain

Ser77 is localized to the PICK1 PDZ domain and thus to the domain that binds the extreme C-termini of PICK1's many interaction partners (Fig. 4A). Although Ser77 is localised away from the binding groove, we wanted to test whether phosphorylation of this residue could affect the binding capacity of the PDZ domain. To test this, we employed our previously described fluorescence polarization binding assay that is based on detection of binding of a fluorescently tagged peptide ligand to purified PICK1 in solution (39). As fluorescently tagged ligand, we used a peptide equivalent to the 13 C-terminal residues of the dopamine transporter (OrG DAT C13), which displays the highest affinity reported for the PICK1 PDZ domain (39). We performed the experiments as competition binding assays using three unlabeled peptides, which are known to bind the PICK1 PDZ domain, i.e. peptides equivalent to the C-termini of DAT (DAT C13), GluA2 (GluA2 C13) and PKC α (PKC α C13), respectively. As a negative control, we used a DAT peptide in which the last residue was changed to aspartate (DAT C13 V to D) which disrupts the interaction with the PICK1 PDZ domain (39). In Fig. 4B, the Y axis shows fluorescence polarization (FP) (arbitrary units, mP), which reflects the rotational diffusion of the fluorescently tagged peptide when bound to PICK1 in solution. Increasing concentration of unlabelled peptide reduces the FP value as the fluorescently labelled tracer is displaced from the larger protein thereby increasing its rotational diffusion. The three peptides (DAT C13, GluA2 C13 and PKC α C13) all inhibited binding of OrG DAT C13 with the same potency in S77D and S77A as in the WT (Fig. 4B and Table 1). Hence, neither removal of the side chain nor mimicking phosphorylation of Ser 77 affected binding of ligand to the PDZ domain.

Phosphorylation of PICK1 by PKC α is not affected by binding of PKC α to the PICK1 PDZ domain

The C-terminus of PKC α is a ligand for the PICK1 PDZ binding pocket (15). Therefore, we investigated whether the possible binding of PKC α to the PICK1 PDZ binding pocket was required for its phosphorylation of PICK1. In this experiment, we used a PICK1 mutant (A87L) that cannot accommodate a peptide in the PDZ binding pocket (39). The PICK1 A87L was phosphorylated to the same level as PICK1 WT ($92\% \pm 14\%$, N.S., $n=4$) (Fig. 5A), indicating that binding of PKC α to the PICK1 PDZ domain is not critical for phosphorylation of PICK1 by the kinase.

Peptide binding to the PDZ domain decreases phosphorylation of PICK1 by PKC α

The above experiment suggests that binding of PKC α to PICK1 is not required for PICK1 phosphorylation; however, the experiment does not address whether occupancy of the PDZ binding crevice *per se* affects the phosphorylation of PICK1 by PKC α . To address this question we preincubated purified PICK1 with either the DAT C13 peptide that binds the PDZ domain with high affinity or the non-binding DAT C13 V to D peptide. Preincubation with DAT C13 resulted in a significant decrease in phosphorylation level of PICK1 compared to preincubation with DAT C13 V to D ($70.5\% \pm 9.6\%$, $p < 0.05$, $n = 3$) (Fig. 5B). The decrease was not due to the simple presence of DAT C13 in the mixture, i.e. no significant difference in phosphorylation level was observed when the non-binding A87L mutation was preincubated with DAT C13 compared to DAT C13 V to D ($95\% \pm 6.9\%$, N.S., $n = 5$) (Fig. 5B). We also analyzed the S77D mutation to assess whether the reduced phosphorylation in the presence of DAT C13 was due to reduced phosphorylation of Ser77. Surprisingly, preincubation with DAT C13 also resulted in a decrease in phosphorylation of PICK1 S77D compared to preincubation with DAT C13 V to D ($75.9\% \pm 6.4\%$, $p < 0.05$, $n = 3$) similar to what was observed for the WT. Taken together, the data suggest that the overall phosphorylation of PICK1 by PKC α is decreased when a peptide is bound to the PICK1 PDZ domain; however this effect is not caused by blocking the phosphorylation of Ser77.

The phosphomimic eYFP-PICK1 S77D displays decreased clustering tendency when overexpressed in COS7 cells

Ser77 is located in close proximity to the lipid binding patch of the PDZ domain (Fig. 3 A) and consequently we wanted to test if mimicking its phosphorylation affected the cellular distribution of PICK. In the experiments we used PICK1 with eYFP fused to the N-terminus (eYFP-PICK1). Fig. 6A shows representative confocal microscopy pictures of the distribution in transiently transfected COS7 cells of eYFP-PICK1 WT and eYFP-PICK1 S77D, respectively. In agreement with previous reports, eYFP-PICK1 formed discrete cytosolic clusters, which have been reported to reflect the membrane binding capacity of PICK1 (14). In the eYFP-PICK1 expressing cells, at least one discrete cytosolic cluster was observed in $73\% \pm 4\%$ of the cells, whereas, for eYFP-PICK1 S77D expressing cells, discrete clusters were observed in $49\% \pm 3\%$ of the cells. Thus, the number of cells containing at least one cluster is significantly reduced for eYFP-PICK1 S77D compared to eYFP-PICK1 (Fig. 6B). The difference in number of cluster containing cells could be related to different expression levels of the two constructs, however, no apparent difference was observed in transfection efficiency and western blots of cell lysates showed similar expression of the two constructs (Fig. 6C.) This immediately suggests that the lipid binding capacity of the phosphomimicking mutant eYFP-PICK1 S77D was reduced. We attempted to test this more directly using *in vitro* lipid vesicle pull down experiments; however, we were not able to reproduce the previously reported vesicle binding capacity of the PDZ domain itself (data not shown) (27). Instead we measured the lipid binding capacity of full length PICK1 in the same assay. In agreement with previous observations (14), we saw robust pull-down of PICK1 by the lipid vesicles but we were unable to detect a difference for full length PICK1 WT and S77D (Fig 6D and E). This suggests that phosphorylation of Ser77 is unlikely to affect the overall lipid binding capacity of PICK1 at least *in vitro*. Moreover, the data suggest that the cytosolic clusters might not be a simple read-out of the lipid binding capacity of PICK1 alone.

DISCUSSION

PICK1 is known to serve multiple functions in relations to its many interaction partners; however, little is known about cellular mechanisms regulating the activity of PICK1. In the

present study, we show that PICK1 is an *in vitro* substrate for two important kinases: PKC α and CaMKII α . We identify a serine at position 77 of the PICK1 PDZ domain as the major PKC α phosphorylation site, at least *in vitro*. In addition, we show evidence that this phosphorylation is crucially regulated by lipid membrane binding and affects the cellular distribution of PICK1, which together are likely to have a functional impact on the interaction partners.

PICK1 was previously shown to be an *in vitro* substrate for PKC α (1), however, it should be noted that Staudinger et al. did the *in vitro* phosphorylation assay with PICK1 coupled to GST, resulting in a protein of similar molecular weight as PKC α . Because PKC α autophosphorylates it cannot be completely excluded that the reported phosphorylation was due to autophosphorylation of PKC α . In our study, the *in vitro* phosphorylation assay was performed with an untagged PICK1 resulting in separation of the two proteins by SDS-PAGE. Hence, it can now be concluded that PICK1 is indeed an *in vitro* substrate for PKC α .

Autophosphorylation was also an issue in the CaMKII α *in vitro* phosphorylation assay since CaMKII α autophosphorylates (45), and PICK1 and CaMKII α have a similar molecular weight. The background signal from CaMKII α autophosphorylation was reduced by preincubating CaMKII α with cold ATP, and the observed increase in phosphorylation upon addition of PICK1 can most likely be ascribed to phosphorylation of PICK1. However, to rule out that the observed effect was due to an effect of PICK1 on CaMKII α autophosphorylation, we also performed the assay with a truncated version of PICK1 (136-416) since this can be separated from CaMKII α by SDS-PAGE. PICK1 136-416 was also phosphorylated by CaMKII α and, thus, we can conclude for the first time that PICK1 is an *in vitro* substrate for CaMKII α and that the phosphorylation at least to some extent takes place beyond residue 135 of PICK1.

The activity of PKC α and CaMKII α are important for synaptic plasticity in which PICK1 also plays a major role. Thus, we speculated that phosphorylation of PICK1 by these kinases can modulate the function of PICK. In this study, we focused on the phosphorylation of PICK1 by PKC α , and by site-directed mutagenesis we identified Ser77 as a major phosphorylation site at least *in vitro*. Unfortunately, our attempt to identify Ser77 as a phosphorylation site in a cellular context failed due to the high lysine/arginine content in the area around Ser77, which prevented isolation of peptides containing Ser77 and their subsequent identification by mass spectrometry. Therefore, we can still only speculate that Ser77 is a phosphorylation site *in vivo*, although we find it likely given the high degree of phosphorylation seen *in vitro* and the high score obtained for the site according to the NetPhos prediction program (41).

To obtain a better understanding of how PKC α phosphorylation of PICK1 might be regulated in a cellular system, we tested the effect of lipid binding on PKC α mediated phosphorylation of PICK1. Performing the phosphorylation assay in the presence of lipid vesicles resulted in approximately 10-fold increase in phosphorylation level of PICK1. Moreover, the vesicle induced increase in phosphorylation was abolished by mutation of Ser77, strongly indicating that the observed effect was due to an increased phosphorylation of Ser77. Thus, recruitment of PICK1 to the membrane might be a key element in promoting the phosphorylation of Ser77. Interestingly, because the lipid vesicles selectively affect phosphorylation of Ser77, the observed increase more likely reflects a conformational change in PICK1 upon lipid binding than a scaffolding of PICK1 and PKC α on the lipid vesicles.

It is also interesting to note that phosphorylation of PICK1 by PKC α was independent of the interaction of the PICK1 PDZ domain and the C-terminus of PKC α , i.e. the PDZ binding

deficient mutant PICK1 A87L was phosphorylated to the same level as WT. This might reflect that PKC α does not bind in the PDZ domain binding groove when phosphorylating PICK1. We observed, however, that binding of a peptide ligand, and thus another putative cellular interaction partner to PICK1, reduced phosphorylation of PICK1. Remarkably, the peptide ligand also reduced phosphorylation of PICK1 when Ser77 was mutated, indicating shielding of another phosphorylation site than Ser77 by the peptide or by a conformational changes of PICK1 that disfavour phosphorylation of another site than Ser77.

Ser77 is located in the PICK1 PDZ domain close to the reported lipid binding patch in the PDZ domain (27) as shown in Fig 3A. This patch was suggested to add to the lipid binding capacity of the BAR domain and thereby to be important for the role of PICK1 in synaptic plasticity (27). The vesicle induced increase in phosphorylation strongly suggests that Ser77 is not docked directly in the membrane; nonetheless, the phosphorylation of Ser77 could be envisioned to decrease the binding capacity of the PICK1 PDZ domain to negatively charged lipid membranes. In agreement with this prediction, we observed that the phosphomimic S77D compromised the clustering tendency of eYFP-PICK1 in COS7 cells. Previous studies have suggested that this clustering tendency represents a measure of PICK1's lipid binding capacity (14). However, we could not reproduce the reported lipid binding of the PICK1 PDZ domain (27) in a vesicle pull-down assay, and the phosphomimic S77D did not reduce the lipid binding capacity of full length PICK1. Nevertheless, it is important to stress that indeed the lipid binding capacity of the PDZ domain could be involved in the cellular localization of PICK1 even though we cannot reproduce it in a simplified *in vitro* assay, especially since the lipid binding of the BAR domain has been shown to be autoinhibited in full length PICK1 (28). Furthermore, the clustering of PICK1 could also reflect oligomerization/polymerization of the protein as seen for other BAR domain proteins (46), which might be important for subcellular localization of PICK1 and its interaction partners. Thus, we speculate that phosphorylation decreases the polymerization of PICK1 in addition to potentially decreasing the lipid binding capacity.

Summarized, the present study identifies PICK1 as a phosphoprotein subject to phosphorylation by major kinases such as PKC α and CaMKII α . Moreover, our data suggest that PKC α mediated phosphorylation is dynamically governed by the interplay between the cellular localisation of PICK1 and occupancy of the PDZ binding pocket (summarized in Fig. 7). Among several possible sites, we identify Ser77 as a main site for PKC α mediated phosphorylation and show how lipid binding enhances phosphorylation of this site, and how this phosphorylation in turn is likely to affect its cellular redistribution. Future studies should further clarify the complex role of phosphorylation in regulating the multiple cellular functions of PICK1.

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Abbreviations

AMPA	α -Amino-3-hydroxy-5-Methyl-4-isoxazolePropionic Acid
ASIC	Acidic Sensing Ion Channel

BAR	Bin/Amphiphysin/Rvs
CaMKIIα	Ca ²⁺ -calmodulin dependent protein Kinase II α
DAT	Dopamine Transporter
eYFP	enhanced Yellow Fluorescent Protein
LTD	Long Term Depression
PDZ	PSD95/Disc large/ZO1
PICK1	Protein Interacting with C-Kinase
PKCα	Protein Kinase C α

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Figure 1. In vitro phosphorylation of PICK1 by PKC α and CaMKII α

The illustrated gels are from representative experiments demonstrating specific phosphorylation of PICK1 by the three kinases, (left) Coomassie stained gels and (right) incorporation of ^{32}P assessed by autoradiography. (A) PKC α *in vitro* phosphorylation of PICK1 WT, control without PICK1 WT is included to show background phosphorylation, n=3 (B) CaMKII α *in vitro* phosphorylation of PICK1 WT, the background from CaMKII α autophosphorylation is minimized by preincubation with unlabelled ATP before addition of PICK1 and ^{32}P -labeled ATP, the right lane on the right gel shows the remaining background, n=3 (C) CaMKII α *in vitro* phosphorylation of PICK1 136-416, n=3

Figure 2. Screening for phosphorylation sites by site directed mutagenesis

(A) Schematic drawing of PICK1 showing the approximate localization of the potential phosphorylation sites together with a table showing the phosphorylation potential score predicted by NetPhos 2.0. Test of the mutants in the PKC α *in vitro* phosphorylation assay (B), CaMKII α *in vitro* phosphorylation assay (C). Upper panels are representative SDS-PAGE showing ^{32}P incorporation and comassie stains. Lower panels show quantitative phosphorylation levels relative to WT. Data are means $\pm$ S.E. of $n=3-5$. Mutation of S77 results in significant decrease of PKC α mediated phosphorylation level (S77A: 48% $\pm$ 7.7%, $n=3$, S77D: 39% $\pm$ 7.7%, means $\pm$ SE $n=4$), * indicates $P<0.05$, and ** indicates $P<0.01$ (one-way ANOVA followed by Dunnett's Multiple Comparison Test). Each n represents a unique protein batch.

Figure 3. Effect of lipid vesicles on PKC α mediated phosphorylation of PICK1

(A) Structural model of the PICK1 PDZ domain highlighting the position of Ser77 (red). Green illustrates the CPC motif in the reported lipid binding patch (Pan et al. *EMBO J* 26(2007)), light blue illustrates the GluA2 ligand in the binding pocket 7. The presented model is based on the coordinates for 2PKU in the PDB database (Pan et al. *EMBO J* 26(2007)). (B) PKC α mediated phosphorylation of purified PICK1 preincubated with vesicles. The phosphorylation level of PICK1 WT without vesicles (control) is defined as 100 %, and the phosphorylation levels of PICK1 WT, PICK1 S77D and PICK1 S77A with vesicles are presented relative to the phosphorylation level of PICK1 WT without vesicles. PICK1 WT: 971% $\pm$ 197%, PICK1 S77D: 53.1% $\pm$ 9.1%, PICK1 S77A: 60.8% $\pm$ 11.6 % (means $\pm$ SE). ** indicates P<0.01 (one way-ANOVA followed by Dunnett's Multiple Comparison Test, n=3). Error bars represent SE values.

Figure 4. Mutating Ser77 does not affect ligand binding affinity of the PICK1 PDZ domain
 (A) Structural model of the PICK1 PDZ domain highlighting the position of Ser77 relative to PDZ binding pocket. Blue illustrates the C-terminus of GluA2 in the PDZ binding groove, red illustrates Ser77 and light purple illustrates the α -helix which is part of the binding pocket and connected to S77. The presented model is based on the coordinates for 2PKU in the PDB database (Pan et al. *EMBO J* 26(2007)). (B), (C), (D) Fluorescence polarization competition binding curves for peptide binding to PICK1 WT, PICK1 S77A and PICK1 S77D. A fixed concentration of Oregon Green labeled DAT peptide (OG DAT C13, ~ 6.7 nM) was incubated together with purified PICK1 and indicated concentrations of unlabeled peptides corresponding to the 13-C-terminal residues of PKC α , GluA2 or DAT C13. DAT C13 (V to D) represents a negative control peptide in which the C-terminal residue was changed from Val to Asp. Data are shown as bound relative to max bound OG DAT C13 (means of triplicates $\pm$ S.E.) and representative of n=3

Figure 5. Phosphorylation of PICK1 by PKC α does not require binding of PKC α to the PICK1 PDZ domain and peptide binding to the PDZ domain decreases phosphorylation

(A) PKC α mediated *in vitro* phosphorylation of PICK1 A87L relative to PICK1 WT. Purified PICK1 WT or A87L were phosphorylated *in vitro* by PKC α , analyzed by SDS-PAGE and phosphorylation quantified as described in *Materials and Methods*. Data are means $\pm$ SE; PICK1 A87L: 92.4 % $\pm$ 14.5 %, n=3. Error bars represent SE. (B) PKC α mediated *in vitro* phosphorylation of PICK1 WT, PICK1 S77D and PICK1 A87L pre-incubated with the DAT-LKV peptide. Data are shown as phosphorylation level normalised to the phosphorylation level of PICK1 WT, PICK1 S77D and PICK1 A87L, respectively, pre-incubated with the non-binding DAT-LKD control peptide (red dashed line). PICK1 WT: 70.5 % $\pm$ 9.6 %, PICK1 S77D: 75.9 % $\pm$ 6.4 %, PICK1 A87L: 94.6 % $\pm$ 6.9 % (means $\pm$ SE, n=3). * indicates P<0.05 in one sample t-test.

Figure 6. Clustering of eYFP-PICK1 in COS7 cells and vesicle binding

(A) Representative pictures showing reduced clustering of eYFP-PICK1 S77D compared to eYFP-PICK1 in transiently transfected COS7 cells. The arrows point to the clusters. (B) Quantification of clustering of WT compared to S77D. Clustering was quantified by counting the fraction of cells with at least one cytosolic cluster of eYFP-PICK1 or eYFP-PICK1 S77D. A cluster was defined as an area with an intensity of 1.5 X the average cytosolic intensity of eYFP. Data are means $\pm$ SE obtained from three independent transfections. At least 10 pictures were taken and approximately 50 cells were counted in each sample. * $P < 0.01$ in two-sided students t-test, $n = 3$, error bars represent SE values. (C) Quantification of the expression level measured by western blot. The level of eYFP-PICK1 was normalized to actin and the level of S77D was calculated to a WT transfected in parallel, $n = 5$ where each n represents an independent transfection. (D) Representative gel of lipid vesicle pull-down of PICK1 WT (left) and PICK1 S77D (right). Purified PICK1 WT or S77D were incubated with lipid vesicles before ultracentrifugation and analysis of pellet (P) and supernatant (S) fraction by SDS-PAGE and western blotting. All pellet was loaded and 1/5 of the supernatant. Ves. = vesicles. (E) Quantification of lipid binding in the *in vitro* vesicle pull-down assay. Data are given as the vesicle bound fraction (means $\pm$ SE, $n = 3$).

The vesicle bound fraction was calculated as
$$\frac{\frac{(\text{Pellet} + \text{ves})}{s}}{\text{Total protein} + \text{ves}} - \frac{\frac{(\text{Pellet} - \text{ves})}{s}}{\text{Total protein} - \text{ves}}$$

Figure 7. Proposed model of regulation of PKC α phosphorylation of PICK1

Our data suggest that PKC α mediated phosphorylation is dynamically governed by the interplay between the cellular localisation of PICK1 and occupancy of the PDZ binding pocket. Very low phosphorylation of PICK1 is expected when it is not bound to membranes and when a peptide ligand is bound in the PDZ domain. Low phosphorylation of PICK1 would be seen when PICK1 neither binds to membrane nor has a PDZ ligand bound to the PDZ binding pocket. Highest phosphorylation of PICK1 would be expected when the protein is bound to membranes with no peptide ligand bound to the PDZ binding pocket. The phosphorylation would be predicted to cause debinding of PICK1 from membrane and likely facilitates its cellular redistribution.

Table 1

Binding affinities for PICK1 WT and PICK1 S77A/D.

	DAT C13	GluA2 C13	PKCα C13	DAT C13 V to D
WT	1.05 [0.987;1.14]	7.6 [6.4;9.1]	49 [38;63]	ND
S77A	1.5 [1.4;1.6]	9.8 [8.0;12]	28 [27;29]	ND
S77D	1.1 [1.0;1.3]	8.8 [8.1;9.6]	29 [25;33]	ND

Data are K_i values in μM (means [SE interval]) calculated from fluorescence polarization competition binding curves. A fixed concentration of Oregon Green labeled DAT peptide (OG DAT C13, ~ 6.7 nM) was incubated together with purified PICK1 WT, PICK1 S77A or PICK1 S77D and increasing concentrations of unlabeled peptides corresponding to the 13-C-terminal residues of PKC α , GluA2 or DAT C13. DAT C13 (V to D) represents a negative control peptide in which the C-terminal residue was changed from Val to Asp. Data were analyzed by non-linear regression analysis as described in Materials and Methods.