Mechanism of A-kinase-anchoring protein 79 (AKAP79) and protein kinase C interaction

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The A-kinase-anchoring protein AKAP79 co-ordinates the location of cAMP-dependent protein kinase, phosphatase 2B (PP2B/calcineurin) and protein kinase C (PKC) at postsynaptic sites in neurons. In this report we focus on the mechanism of interaction between AKAP79 and PKC. We show that neither lipid activators nor kinase activation are required for association with AKAP79. The anchoring protein binds and inhibits the conserved catalytic core of PKCβII. AKAP79 also associates with conventional, novel and atypical isoforms of PKC *in vitro* and *in vivo*, and immunofluorescence staining of rat hippocampal neurons demonstrates that the murine anchoring-protein homologue AKAP150 is co-distributed with PKCα/β, PKCϵ or PKCι. Binding of the AKAP79(31–52) peptide, which inhibits kinase activity, exposes the pseudosubstrate domain of PKCβII, allowing endoproteinase Arg-C proteolysis in the absence of kinase activators. Reciprocal experiments have identified two arginine residues at positions 39 and 40 that are essential for AKAP79(31–52) peptide inhibition of PKCβII. Likewise, the same mutations in the full-length anchoring protein reduced inhibition of PKCβII. Thus AKAP79 associates with multiple PKC isoforms through a mechanism involving protein–protein interactions at the catalytic core where binding of the anchoring protein inhibits kinase activity through displacement of the pseudosubstrate.

Key words: anchoring protein, postsynaptic density, signal transduction, subcellular localization.

INTRODUCTION

Specific phosphorylation of intracellular substrates is important for the control of many aspects of cellular function [1]. Protein phosphorylation is a tightly regulated process involving the concerted action of protein kinases and phosphatases. Recently, targeting of these signalling enzymes to precise subcellular locations has emerged as a mechanism for tight control of phosphorylation events within a defined microenvironment [2,3]. Compartmentalization of protein kinases and phosphatases may also increase the speed and fidelity of specific substrate phosphorylation and dephosphorylation in response to particular second-messenger signals. This is achieved, in part, through interactions with anchoring and targeting proteins [4]. A growing number of these proteins has now been identified, which bind protein kinases and phosphatases specifically and frequently regulate their location and activity [3,5]. For example, A-kinase-anchoring proteins (AKAPs) bind to the regulatory subunit (RII) of the cAMP-dependent protein kinase (PKA) and direct the kinase to discrete subcellular structures [6,7].

The neuronal anchoring protein, AKAP79, is enriched in postsynaptic fractions, and immunostaining in hippocampal neurons reveals a concentration at the periphery of the cell body and in neurite extensions [8–10]. AKAP79 targeting has recently been shown to be mediated through three basic-rich regions that bind acidic phospholipids and which appear to be regulated by phosphorylation and calmodulin binding [11]. Whereas AKAP79 was originally cloned as an RII-binding protein, it has also been shown to associate with protein phosphatase 2B calcineurin and protein kinase C (PKC) [10,12]. Thus AKAP79 directs three signalling enzymes to postsynaptic sites and has been proposed to function as a signalling scaffold co-ordinating the phosphorylation state of synaptic proteins [13]. However, there are still questions surrounding mechanisms of interaction and regulation of this signalling complex. Each enzyme associated with the anchoring protein is maintained in an inactive state: the catalytic (C) subunit of PKA through interactions with anchored RII, whereas calcineurin and PKC bind through direct interactions with the anchoring protein [10,12]. The catalytic subunit of PKA is released as freely active enzyme following cAMP binding to RII, whereas the regulatory mechanism for calcineurin is unknown. We have shown previously that Ca^{2+}–calmodulin antagonizes the interaction between AKAP79 and PKC, releasing the inhibited kinase from its association with the anchoring protein [14]. However, the precise mechanism for the interaction of AKAP79 and PKC is not fully understood.

In this study we have focused on the association of PKC with the anchoring protein and have extended our understanding of the mechanism of PKC–AKAP79 interaction in three ways. First, PKC binds to the anchoring protein in a manner that does not require lipid cofactors or activation of the kinase. Secondly, the catalytic core of the kinase provides a principal site of contact with AKAP79. Finally, a pair of arginine residues at positions 39 and 40 on AKAP79 participate in binding and inhibition of PKC in a mechanism involving the pseudosubstrate.

EXPERIMENTAL

Bacterial expression and purification of recombinant AKAP79 proteins

AKAP79 was expressed as an N-terminal His-tagged fusion protein in pET16b (Novagen) in the BL21/DE3 strain of *Escherichia coli* and purified using Ni-affinity chromatography

Abbreviations used: AKAP, A-kinase-anchoring protein; PKC, protein kinase C; DG, diacylglycerol; PS, phosphatidylserine; PA, phosphatidic acid; PKA, cAMP-dependent protein kinase; GST, glutathione S-transferase; DTT, dithiothreitol; TBS, Tris-buffered saline.

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(Pharmacia). For construction of the AKAP79 R39,40A mutant, expression vectors encoding the cDNA sequence of AKAP79 with mutation of Arg-39 and Arg-40 to Ala were made by quick-change site-directed mutagenesis (Stratagene) using wild-type AKAP79 in pET16b as template. Synthetic oligonucleotide primers were designed to introduce four nucleotide base changes (shown here in bold): the nucleotide sequences of the sense and antisense primers were GCATCCATGCTTTGCTTCAAG**GC**-A**GC**AAAGAAAGCAGCCAAAGCAC and GTGCTTTGGC-TGCTTTCTTT**GCTGC**CTTGAAGCAAAGCATGGATGC. The PCR product was digested with *Dpn*I and transformed into DH5a-competent cells. The sequence was verified by DNA sequencing.

Expression of PKC isoforms and mutants

Sf21 insect cells were infected with high-titre (1×10^8 plaque-forming units·ml^{-1}) baculovirus encoding wild-type PKC isoforms (α, βII, δ, ϵ, ζ and θ) or mutants of PKC βII (GST–PKCβII kinase domain [15], K371R, T500V and D248R/D254R; where GST is glutathione S-transferase), as described in [16]. The cells were harvested after 3 days at 27 °C, lysed by homogenization in buffer containing 50 mM Hepes (pH 7.4), 0.2 % Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), 85 μM leupeptin, 2 mM benzamidine and 0.2 mM PMSF, and centrifuged at 100000 ***g*** for 20 min at 4 °C. Experiments were performed with the supernatant (detergent-soluble) diluted 2-fold in glycerol and stored at −20 °C.

Immunoprecipitation of AKAP79

AKAP79 (2 μg) was incubated with detergent-soluble extracts (100 μg) of Sf21 insect cells expressing PKC in hypotonic buffer [10 mM Hepes, pH 7.4, 1.5 mM $MgCl_2$, 10 mM KCl, Nonidet P-40 (0.2 % w/v), 1 mM DTT, 2 mM $CaCl_2$, 20 μg/ml phosphatidylserine (PS), 0.1 % BSA, 1 mM benzamidine, 2 μg/ml leupeptin, 2 μg/ml pepstatin and 100 mM 4-(2-aminoethyl)-benzenesulphonyl fluoride hydrochloride] for 1 h at 4 °C. AKAP79 was immunoprecipitated using an AKAP79-specific antibody (918I from Icos, Seattle, WA, U.S.A.) followed by detection of various PKC isoforms by Western blotting as described in [14]. For immunoprecipitation in the presence of different lipids, PS (20 μg/ml) was substituted with 140 μM PS, 140 μM phosphatidic acid (PA), 140 μM PS/4.0 μM diacylglycerol (DG; all from Avanti), or 100 nM phorbol ester (PMA; Sigma). Lipid solutions were prepared from chloroform stock solutions by drying the solutions with nitrogen and resuspending the lipid in 20 mM Hepes (pH 7.4) and sonicating for 30 s. For immunoprecipitation of AKAP79 with PKCβII mutants, Western-blot analysis was performed with a monoclonal antibody to PKCα/β (Transduction Laboratories). For immunoprecipitation of AKAP79 with different PKC isoforms, AKAP79 was incubated with Sf21 cells expressing PKCα, PKCβII, PKCδ, PKCϵ, PKCθ and PKCζ. Western-blot analysis was performed with isoform-specific antibodies (α, δ, ϵ, θ and ι from Transduction Laboratories; ζ and βII from Santa Cruz Biotechnology). AKAP79 (10 μg) was also immunoprecipitated with Sf21 cells expressing the GST-tagged catalytic core of PKC (100 μg).

PKC activity assay

PKCβII was assayed as described in [14]. PKCβII catalytic-domain activity was assayed in the presence of 2 mM EGTA and 0.1 % (w/v) Triton X-100. Dose–response curves were generated over an inhibitor concentration range of 0.1–10 μM for AKAP79(31–52) and AKAP79(31–52) (R39,40A) peptides and 0.1–5 μM for AKAP79 wild-type and full-length AKAP79 (R39,40A) mutant proteins.

PKCβII proteolysis

Limited trypsin digestion of PKCβII was conducted as described in [14]. Proteolytic products were then assayed for PKC activity in the presence of PKC activators: 300 μM $CaCl_2$/0.1 % (w/v) Triton X-100 micelles containing 20 mol % PS and 5 mol % dioleoylglycerol (Avanti). Limited endoproteinase Arg-C digestion of PKCβII was performed as described in [17] in a total volume of 80 μl (containing 20 mM Hepes, pH 7.5, 2 mM DTT, 300 μM $CaCl_2$, 140 μM PS and 3.8 μM DG) in the presence of 8 units/ml endoproteinase Arg-C. For experiments with peptides, PKCβII was pre-incubated with 100 μM AKAP79(31–52) or 100 μM AKAP79(31–52) (R39,40A). The reaction was stopped by the addition of 25 μl of sample-preparation buffer, and proteolytic fragments were separated by SDS/PAGE (9 % gels) and transferred on to nitrocellulose. Immunoblots were probed with antibodies to PKCβII (Santa Cruz Biotechnology).

PKC overlay

PKC binding was assayed by modification of the overlay assay described by Hyatt et al. [18]. Proteins were separated by SDS/PAGE and transferred on to nitrocellulose. Filters were washed three times with Tris-buffered saline (TBS; 10 mM Tris, pH 7.6, 140 mM NaCl), blocked in Blotto (TBS with 5 % non-fat dry milk and 0.1 % BSA) for 30 min, and washed three times with TBS. Filters were then incubated with PKC (titrated amounts of PKC isoforms according to expression levels of the Sf21 cell extracts) in assay buffer [TBS containing 1 % BSA, 1.2 mM $CaCl_2$, 1 mM EGTA, 20 μg/ml PS, 1 mM benzamidine, 2 μg/ml leupeptin, 2 μg/ml pepstatin and 100 mM 4-(2-aminoethyl)benzenesulphonyl fluoride hydrochloride] for 1 h. Filters were again washed three times with TBS, and incubated with isoform-specific antibodies. For overlays conducted in the presence of peptides, PKC was pre-incubated in assay buffer with 2.0 μM peptide for 1 h at 4 °C.

Co-purification of AKAP150 and PKC using cAMP–agarose

Rat brain extracts were prepared by grinding frozen Sprague–Dawley unstripped rat brains (Pelfreze) in liquid nitrogen, followed by Dounce homogenization in lysis buffer [20 mM Hepes, pH 7.4, 20 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.5 % Triton X-100, 1 mM DTT, 1 mM benzamidine, 2 μg/ml leupeptin, 2 μg/ml pepstatin and 100 mM 4-(2-aminoethyl)-benzenesulphonyl fluoride hydrochloride] and centrifugation at 15000 ***g*** for 30 min at 4 °C. Experiments were performed with pre-incubation of supernatant in the absence and presence of 50 mM cAMP (Sigma). Extracts were incubated with cAMP–agarose for 18 h at 4 °C. Following incubation, the resin was washed twice with hypotonic buffer [10 mM Hepes, pH 7.4, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1 % Nonidet P-40, 1 mM DTT, 1 mM benzamidine, 2 μg/ml leupeptin, 2 μg/ml pepstatin, and 100 mM 4-(2-aminoethyl)-benzenesulphonyl fluoride hydrochloride] containing 0.5 M NaCl, and four times with hypotonic buffer without NaCl. Proteins were eluted from the resin by incubation with 75 mM cAMP for 1 h at room temperature. Eluted proteins were precipitated with 6 % trichloroacetic acid and resuspended in SDS-sample preparation buffer. Proteins

were separated by SDS/PAGE on a 4–15% gradient gel and transferred on to nitrocellulose. Filters were analysed by Western blotting with PKC-isoform-specific antibodies and an antibody to AKAP150 (4361J).

Immunocytochemistry

Cultured neonatal hippocampal neurons were fixed and permeabilized as described in [14]. Cells were blocked in PBS containing 0.2% BSA for 1 h and then incubated with mixtures containing affinity purified rabbit anti-AKAP150 antibody 4361J and mouse anti-PKCα, anti-PKCϵ or anti-PKCι or affinity purified goat anti-RII. Coverslips were washed three times with PBS containing 0.2% BSA and incubated with a mixture of FITC-conjugated anti-rabbit (1:200), Texas Red-conjugated anti-mouse (1:200) and Cy5-conjugated anti-goat (1:500) secondary antibodies (all from Jackson) in PBS containing 0.2% BSA. The coverslips were then washed three times in PBS containing 0.2% BSA, washed in Nanopure water and mounted with Prolong (Molecular Probes). Cells were analysed with a Bio-Rad confocal microscope [19].

RESULTS

Interaction of AKAP79 and PKCβII does not require lipid activators

We have shown previously that PKCβII associates with AKAP79 in a Ca^{2+}-independent manner and in the presence of PS [10,14]. However, the precise activation state of the kinase when bound to the anchoring protein is not known. Activation of PKCβII requires DG, PS and Ca^{2+} [20]. In addition, we have shown recently that sub-membrane targeting of AKAP79 involves acidic phospholipids that bind to distinct regions on the anchoring protein, including PtdIns(4,5)P_2 and PS [11]. In order to assess whether lipid bridging mediated AKAP79–PKC interactions, the anchoring protein was incubated with PKCβII and co-precipitation experiments were performed in the presence or absence of lipid and activators (Figure 1). Immunoblot analysis demonstrates that PKCβII co-immunoprecipitated with AKAP79 independently of the presence of PS or PA (Figure 1A). Control experiments with pre-immune sera did not detect the enzyme (Figure 1A). Significantly, PKCβII co-precipitated when lipid was not present (Figure 1A). The kinase also co-precipitated with AKAP79 in the presence of both PS/DG and PMA (Figure 1B) as well as with PA/DG (results not shown). These results establish that lipid is not required for interaction of PKC and AKAP79 and suggest that the activation state of PKC is not significant for association with the anchoring protein.

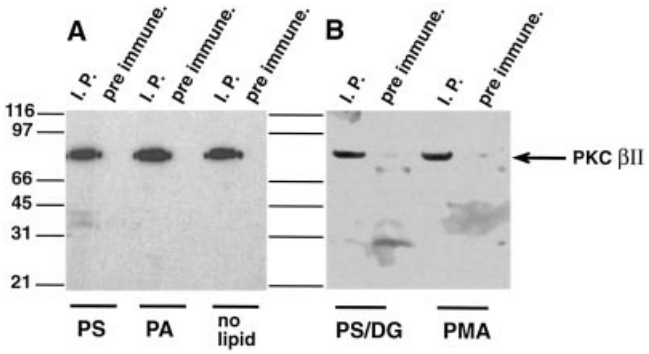

Figure 1 AKAP79 binding to PKCβII does not require lipid activators

AKAP79 (2 μg) and soluble Sf21 cell lysate (100 μg) expressing PKCβII were incubated in the presence of 140 μM PS, 140 μM PA or no lipid (**A**), and 140 μM PS/4 μM DG or 0.1 μM PMA (**B**) for 1 h at 4 °C. Proteins were immunoprecipitated with a polyclonal antibody to AKAP79 918I (I. P.) or preimmune serum as described in the Experimental section. Precipitated proteins were immunoblotted and detected with a monoclonal antibody to PKCα/β. The migration of PKCβII is indicated. Shown is a representative of at least three separate experiments. Molecular-mass markers are indicated in kDa.

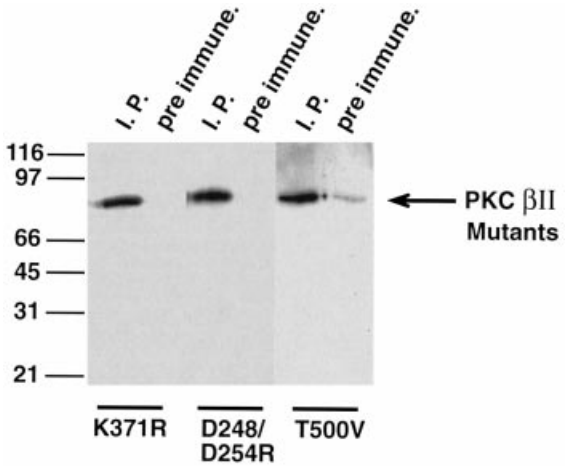

Figure 2 AKAP79 binds PKCβII activation-site mutants

AKAP79 (2 μg) and soluble Sf21 cell lysate (100 μg) expressing PKCβII mutants K371R, D248R/D254R and T500V were incubated for 1 h at 4 °C. Proteins were immunoprecipitated with a polyclonal antibody to AKAP79 918I (I. P.) or preimmune sera as described in the Experimental section. Precipitated proteins were immunoblotted and probed with a monoclonal antibody to PKCα/β. Shown is a representative of at least three separate experiments. Molecular-mass markers are indicated in kDa.

PKCβII does not need to be in an active conformation to bind AKAP79

In order to investigate further the mechanism of PKCβII binding to AKAP79, co-precipitation experiments were performed with mutants of PKCβII. Mutation of the invariant Lys residue in the ATP-binding site of Ser/Thr kinase catalytic domains renders the kinase inactive [21]. Mutation of this essential Lys to Arg in PKCβII (K371R) did not abolish binding to AKAP79 (Figure 2). Other studies have shown that phosphorylation at the activation loop is required for cofactor generation of the mature activatable conformation of PKCβII [22,23]. However, mutation of the activation-loop phosphorylation site, Thr-500 to Val (T500V) in PKCβII, did not abolish the interaction with AKAP79 (Figure 2). We also investigated the effect of mutations in the regulatory C2 domain (the Ca^{2+}-dependent PS-binding domain) of PKCβII on AKAP79 binding. Replacement of two acidic residues with two basic charges in the Ca^{2+}-binding site of PKCβII (D248R/D254R) decreases Ca^{2+} binding as well as anionic-lipid affinity for PKCβII [16]. This C2-domain mutant also co-precipitated with AKAP79 (Figure 2), consistent with previous results showing that Ca^{2+} is not required for PKCβII binding to the anchoring protein [14]. Collectively, the data presented in Figures 1 and 2 suggest that PKCβII binds to AKAP79 regardless of the presence of lipid activators of PKC and that the active conformation of the enzyme is not important for the interaction.

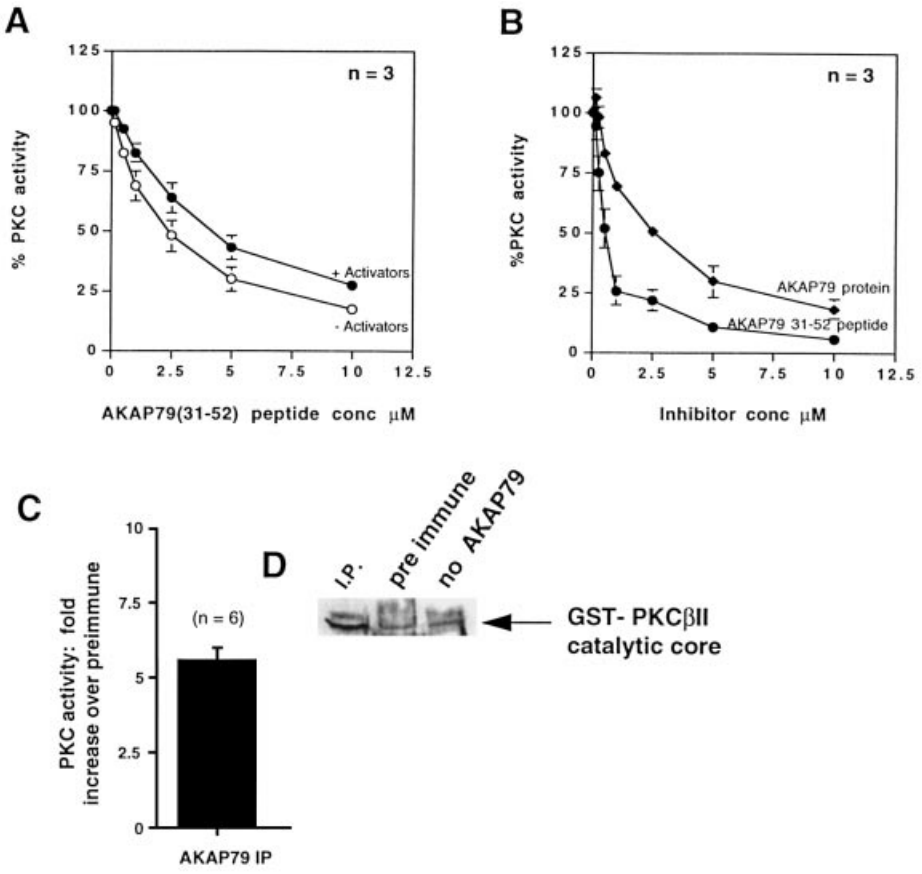

Figure 3 AKAP79 binds PKCβII catalytic core

PKC (30 nmol) was digested with trypsin (300 ng/ml) to generate a constitutively active enzyme, as described in the Experimental section. (**A**) Dose-response curves of activator-independent PKCβII activity in the presence of increasing concentrations of AKAP79(31–52) peptide assayed in the presence (●) and absence (○) of PKC activators (PS/DG and Ca^{2+}). Values shown are mean ± S.E.M. ($n = 3$). (**B**) Dose–response curve of the activity of a recombinant PKCβII catalytic-domain fragment assayed in the presence of increasing concentrations of AKAP79(31–52) peptide (●) and AKAP79 protein (◆). Values shown are mean ± S.E.M. ($n = 3$). (**C**) AKAP79 (10 μg) was incubated with soluble Sf21 cell lysate (100 μg protein) expressing GST–PKCβII catalytic domain for 1 h at 4 °C. (**D**) Proteins were immunoprecipitated with a polyclonal antibody to AKAP79 918I (I. P.) or preimmune serum.

AKAP79 interacts with PKCβII catalytic core

Further mapping studies were conducted to determine which regions of the kinase were required for association with AKAP79 [10]. Trypsin digestion of PKCβII removes the regulatory domain of the kinase to generate a constitutively active catalytic core [24], as evidenced by similar activities in the presence or absence of PKC activators (Figure 3A). This trypsin-generated catalytic fragment of PKCβII was inhibited to the same extent as the intact enzyme by a peptide derived from AKAP79, residues 31–52 [10] (Figure 3A). This peptide has previously been shown to act as a competitive inhibitor of kinase activity and block the AKAP79–PKC interaction *in vitro* [10]. Likewise, the enzymic activity of a GST–PKCβII catalytic-domain fusion protein was inhibited by both the AKAP79(31–52) peptide and the full-length anchoring protein in a dose-dependent manner, with IC_{50} values of 0.9 ± 0.3 ($n = 4$) and 2.7 ± 0.2 μM ($n = 3$) respectively (Figure 3B). To look more directly at AKAP79 binding to the catalytic domain, we performed co-precipitation experiments with Sf21 cell extracts expressing the GST–PKCβII catalytic-domain fragment and incubated with recombinant AKAP79 (Figure 3C). In these experiments, a $5.6(\pm 0.4)$-fold increase ($n = 6$) in activator-independent PKC activity over control was measured in material immunoprecipitated with an antibody to AKAP79 (Figure 3D). Complementary experiments demonstrated that the GST–PKCβII catalytic domain co-immunoprecipitated with AKAP79, but not with preimmune sera or when AKAP79 was not added (results not shown). Taken together, these results suggest that AKAP79 interacts with the catalytic domain of PKCβII.

AKAP79 interacts with conventional, novel and atypical isoforms of PKC

In light of our binding and inhibition studies, it is clear that AKAP79 associates with sites in the catalytic core of PKCβII. As all PKC isoforms exhibit a high degree of conservation in the kinase catalytic core, we used two reciprocal approaches: PKC overlay analysis and co-precipitation of AKAP79–PKC complexes to determine which additional PKC isoforms bind the anchoring protein *in vitro* (Figure 4). Solid-phase overlay assays, using different isoforms of PKC as probes, demonstrate that AKAP79 binds conventional isoforms, PKCα and βII, as well as novel isoforms, PKCδ and ϵ, and atypical PKCζ (Figure 4A). In separate experiments, solution-phase interaction of AKAP79 with individual PKC isoforms was confirmed by immunoprecipitation of the anchoring-protein–PKC complex from Sf21 cell

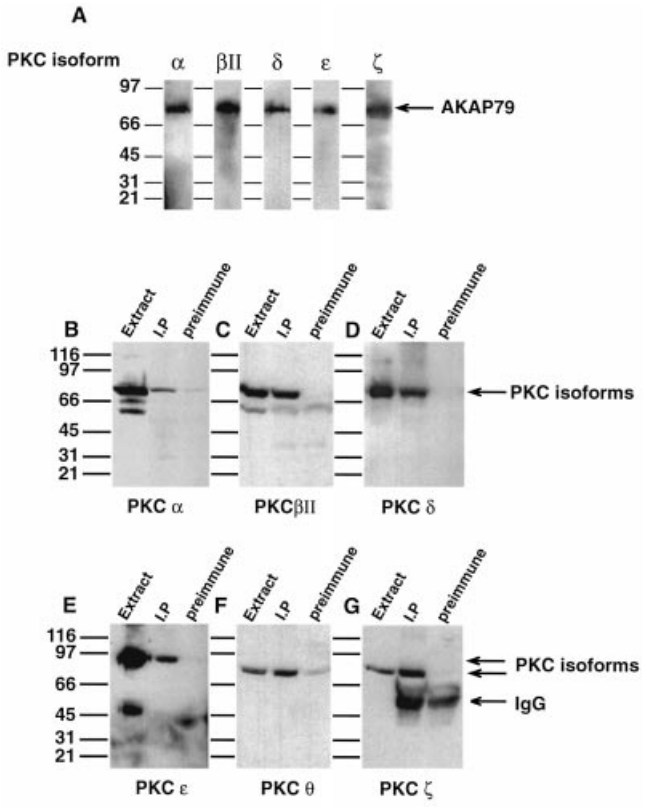

Figure 4 AKAP79 binds multiple PKC isoforms *in vitro*

(**A**) PKC overlays. AKAP79 (2 μg) was separated by SDS/PAGE and transferred on to nitrocellulose. Filters were probed with soluble Sf21 cell lysates expressing PKCα, PKCβII, PKCδ, PKCϵ and PKCζ and overlay binding detected using isoform-specific antibodies as described in the Experimental section. Each PKC isoform is denoted above each lane and the migration position of AKAP79 is indicated. (**B**–**G**) Co-precipitation of AKAP79 and PKC isoforms. AKAP79 (2 μg) was incubated with PKC isoforms, soluble Sf21 cell lysates (100 μg of protein) expressing PKCα, PKCβII, PKCδ, PKCϵ, PKCθ or PKCζ, for 1 h at 4 °C. Proteins were immunoprecipitated with a polyclonal antibody to AKAP79 918I (I. P.) or preimmune serum. Precipitated proteins were immunoblotted and probed with antibodies to the specific PKC isoforms: (**B**) PKCα, (**C**) PKCβII, (**D**) PKCδ, (**E**) PKCϵ, (**F**) PKCθ or (**G**) PKCζ as described in the Experimental section. Each PKC isoform is denoted. Shown is a representative of at least three separate experiments. Molecular-mass markers are indicated in kDa.

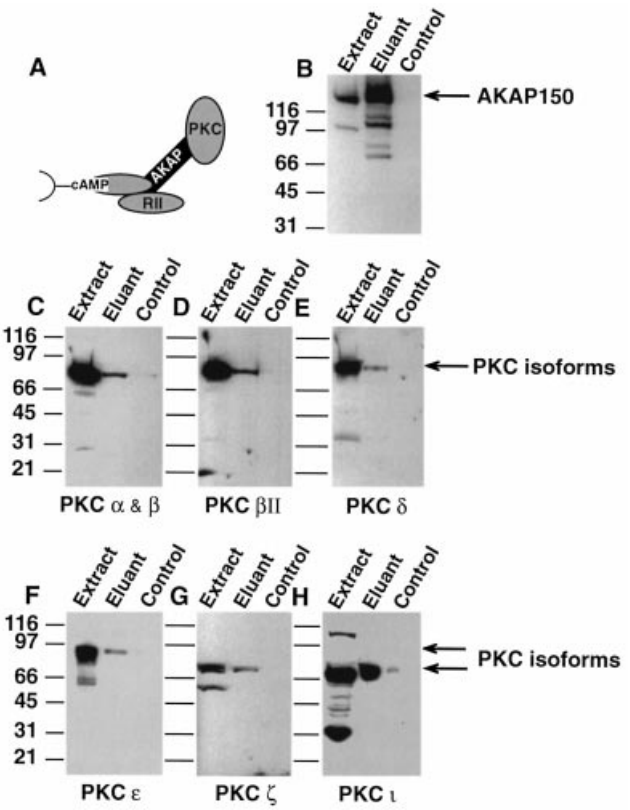

Figure 5 Co-purification of AKAP150, the rat homologue of AKAP79, with PKC isoforms from rat brain extracts

(**A**) A schematic representation of cAMP–agarose co-purification of RII, AKAP and PKC. Rat brain extracts were incubated with cAMP–agarose and co-purifying proteins eluted with cAMP and immunoblotted as described in the Experimental section. Eluant refers to specific elution with cAMP. Control experiments were performed by pre-incubating extracts in the presence of excess cAMP. Immunoblots were probed with antibodies to AKAP150 4361J (**B**), PKCα/β (**C**), PKCβII (**D**), PKCδ (**E**), PKCϵ (**F**), PKCζ (**G**) and PKCι (**H**). The migration position of AKAP150 is indicated. Shown are representatives of at least three separate experiments. Molecular-mass markers are indicated in kDa.

extracts expressing PKCα, PKCβII, PKCδ, PKCϵ, PKCθ or PKCζ (Figures 4B–4G respectively). Immunoblots probed with the appropriate PKC antibody specifically detected co-precipitation of each isoform with AKAP79 (Figures 4B–4G). Preimmune sera were used as a control to confirm that individual PKC isoforms did not non-specifically bind to IgG (Figures 4B–4G). In addition, AKAP79 does not bind the free catalytic subunit of PKA [10], suggesting a specific interaction with PKC isoforms.

While it is clear that AKAP79 binds all classes of PKC isoforms *in vitro*, it was important to determine which isoforms interact with the anchoring protein inside cells. In order to conduct these experiments, we took advantage of the fact that AKAP79 also interacts with the PKA holoenzyme [8]. We used affinity purification of the regulatory subunit of PKA on cAMP–agarose to co-purify AKAP150 (the rat homologue of human AKAP79) and PKC from rat brain extracts (Figure 5A). Initial experiments confirmed that AKAP150 was specifically eluted from cAMP–agarose in buffers containing excess cAMP (Figure 5B). Co-purification of individual PKC isoforms from rat brain was detected using isoform-specific antibodies. PKCα/β, PKCβII, PKCδ, PKCϵ, PKCζ and PKCι (Figures 5C–5H respectively) were eluted selectively from the resin with excess cAMP. Control experiments confirmed that the anchoring protein complex was specifically interacting with the cAMP ligand, as no AKAP150 or PKC was recovered from extracts pre-incubated with the affinity resin in the presence of excess cAMP (Figures 5C–5H). Importantly, these experiments show that the rat homologue of AKAP79 interacts with various PKC isoforms in the brain.

AKAP79 is a neuronal protein that is highly expressed in certain brain regions and present in postsynaptic density fractions [8,9]. It has been shown previously that AKAP150, the rat homologue of AKAP79, is concentrated at the periphery of neuronal cell bodies and in discrete dendritic regions of neurons [9,10]. Therefore, we took an immunocytochemical approach to examine the subcellular distribution of AKAP150, RII and selected PKC isoforms in cultured rat hippocampal neurons. As expected, AKAP150 is present at the periphery of the cell body and exhibits a punctuate distribution in neurite extensions (Figures 6A, 6D, 6G and 6J). The staining patterns of each PKC isoform examined were distinct, but overlapped with the ancho-

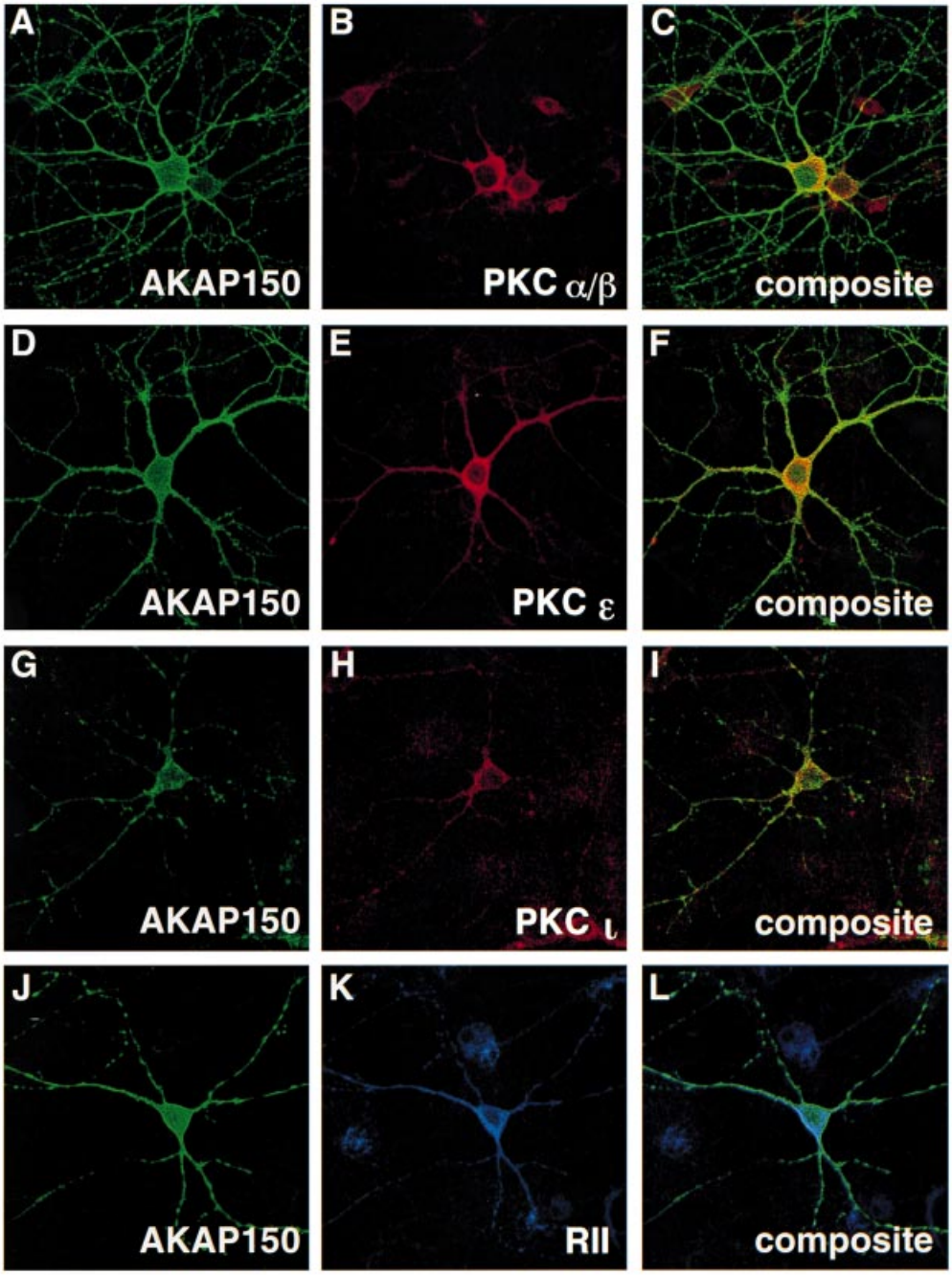

Figure 6 Subcellular distribution of AKAP150 and selected PKC isoforms in neurons

Neonatal rat hippocampal neurons were fixed with 3.7% formaldehyde and permeabilized with 100% acetone at −20 °C for 1 min. Cells were incubated with antibodies to AKAP150 (affinity-purified rabbit 4361J) and PKCα/β, PKCϵ or PKCι (mouse monoclonal antibodies). Control experiments detected the regulatory subunit of PKA, RII (affinity-purified goat anti-RII antibodies). Protein staining was detected with FITC-conjugated anti-rabbit (1:200), Texas Red-conjugated anti-mouse (1:200) and Cy5-conjugated anti-goat (1:500) secondary antibodies. Images were analysed by confocal microscopy at ×63 magnification, as described in the Experimental section. (**A**, **D**, **G**, **J**) AKAP150; (**B**) PKCα/β; (**E**) PKCϵ; (**H**) PKCι; and (**K**) RII. (**C**, **F**, **I**, **L**) Double-label immunofluorescent composite images were analysed in the same focal plane.

ring protein (Figures 6C, 6F and 6I). The conventional isoforms PKCα and PKCβ were concentrated in the neuronal cell body and excluded from the nucleus, with less staining in neuronal extensions (Figure 6B). The non-conventional isoform PKCϵ was largely in the cell body, but there was also defined staining in dentrite extensions (Figure 6E). The atypical isoform PKCι appeared to be concentrated in the cell body and was present to a lesser extent in neuronal extensions with a more punctuate distribution (Figure 6H). Composite images show that there is some overlap in the staining pattern of PKC isoforms α/β, ϵ or ι with AKAP150 (Figures 6C, 6F and 6I). Analysis of confocal images suggests that each PKC isoform and AKAP150 occupy the same focal plane, particularly in the cell body of each neuron (Figures 6C, 6F and 6I). These observations are consistent with

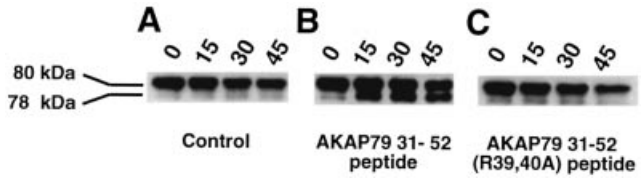

Figure 7 AKAP79(31–52) peptide exposes the pseudosubstrate domain of PKCβII to Arg-C proteolysis

PKCβII was digested with endoproteinase Arg-C as described in the Experimental section. Experiments were performed in the absence of PKC activators and (**A**) in the absence of AKAP79 peptides, (**B**) in the presence of AKAP79(31–52) peptide, (**C**) in the presence of AKAP79(31–52) (R39,40A) peptide. Shown is a time course of Arg-C digestion at 0, 15, 30 and 45 min. PKCβII was detected by immunoblot using an antibody to PKCβII. Shown is a representative of at least three separate experiments.

the co-distribution of both proteins and support our data on the isolation of PKC/AKAP complexes from rat brain. However, there are clearly regions of distinct staining for each PKC isoform. These findings are analogous to the situation with type-II PKA anchoring, where RII is concentrated in the cell body and present in neurite extensions, but is excluded from the nucleus (Figures 6J–6L). Also, the composite image (Figure 6L) is consistent with biochemical evidence that RII and AKAP150 associate in neurons [8,9,25]. Collectively, immunocytochemical staining of AKAP150, RII and the PKC isoforms demonstrates that a fraction of these proteins is co-distributed in neurons and is consistent with the *in vitro* and *in vivo* co-purification experiments described above.

Binding of AKAP79(31–52) peptide to PKCβII exposes the pseudosubstrate sequence allowing Arg-C proteolysis in the absence of PKCβII activators

In an effort to understand the mechanism of AKAP79 interaction with the catalytic core of PKC more precisely, we used endoproteinase Arg-C digestion of PKCβII. Previous studies have shown that limited proteolysis of PKCβII by endoproteinase Arg-C, in the presence of PKC activators, cleaves the pseudosubstrate region to generate a 78-kDa form [17]. This corresponds to the cleavage of Arg-19 within the autoinhibitory pseudosubstrate region of PKCβII. Mechanistic studies suggest that upon activation the enzyme undergoes a conformational change that releases the pseudosubstrate, allowing substrate access to the active site [26]. Given that the AKAP79(31–52) peptide inhibits PKCβII activity, we were interested in determining if this peptide bound PKCβII with a similar mechanism to the pseudosubstrate. As expected, PKCβII was not sensitive to Arg-C in the absence of PKC activators (Figure 7A), but the 78-kDa fragment was generated when the experiments were performed in the presence of activators (results not shown). Interestingly, incubation of PKCβII with the AKAP79(31–52) peptide promotes the formation of a 78-kDa fragment in the absence of activators, indicating exposure and cleavage of the pseudosubstrate (Figure 7B). Thus it appears that the AKAP79(31–52) peptide binds to PKCβII in a way that exposes Arg-19. On the basis of these findings we propose that the AKAP79 peptide binds at or close to the substrate-binding groove in the catalytic core of the kinase to displace the pseudosubstrate.

Molecular modelling of the catalytic core of PKC indicates that Arg-19 is surrounded by a cluster of acidic residues when the pseudosubstrate is bound to the substrate-binding site [27]. Interestingly, the AKAP79(31–52) peptide resembles the pseudosubstrate, as it is rich in basic residues and inhibits PKC activity, consistent with binding at this site [10]. In addition, this peptide mimics activator-dependent digestion of PKCβII by Arg-C, indicating that peptide binding results in a conformational change, presumably by competing with the pseudosubstrate (Figure 7B). To address the importance of basic residues in AKAP79 binding to PKC, we replaced two Arg residues (Arg-39 and Arg-40) with Ala in the (31–52) peptide (see Figure 8A). The AKAP79(31–52) (R39,40A) peptide did not result in significant digestion of PKCβII by Arg-C in the absence of activators (Figure 7C). Thus, it appears that the arginine pair within the AKAP79(31–52) peptide are essential for exposing the pseudosubstrate to cleavage by endoproteinase Arg-C.

Arg-39 and Arg-40 are important for AKAP79 inhibition of PKCβII

To investigate further the role of Arg-39 and Arg-40 for binding of PKC (Figure 8A), we tested the ability of the modified (31–52) (R39,40A) peptide to block PKCβII binding by overlay. The (31–52) (R39,40A) peptide did not block PKCβII binding to AKAP79 (Figure 8B, right panel), whereas the wild-type peptide was able to block PKC binding (Figure 8B, left panel). In addition, the AKAP79(31–52) (R39,40A) peptide was a poor inhibitor of PKCβII activity (Figure 8C, ●), whereas the wild-type AKAP79(31–52) peptide was a potent inhibitor of kinase activity (Figure 8C, ○). To rule out the possibility that this inhibition was due to PKC preferentially binding any basic peptide, additional control experiments were performed with a peptide of similar basic charge but of different sequence from the (31–52) peptide. This randomized peptide was unable to inhibit the kinase (Figure 8C, □). In order to investigate the effect of Arg-39 and Arg-40 on PKC binding and inhibition in the context of the full-length AKAP79 protein, we mutated both residues to alanine. The AKAP79 (R39,40A) mutant protein shifted the dose response of inhibition when compared with the wild-type anchoring protein (Figure 8D). At an inhibitor concentration of 5 μM, PKCβII activity was reduced to 10 % with the wild-type anchoring protein but only to 50 % with the AKAP79 (R39,40A) mutant (Figure 8D). The partial effects observed with the full-length AKAP79 (R39,40A) mutant suggest that additional determinants for interaction with PKCβII reside within the anchoring protein. This idea was confirmed by co-immunoprecipitation of the AKAP79 (R39,40A) mutant with PKCβII (Figure 8E). Collectively, these results demonstrate that basic residues Arg-39 and Arg-40 within the AKAP79 PKC-binding region are important for inhibition and binding in the (31–52) peptide sequence and influence inhibition of PKCβII activity in the context of the full-length anchoring protein.

DISCUSSION

In this study we have extended our analysis of AKAP79–PKC interaction in order to understand the mechanism of these protein–protein interactions. There are now an increasing number of PKC-binding proteins believed to play a role in directing the location of individual PKC isoforms to particular subcellular locations (reviewed in [4,5]). These include: substrate-binding proteins that interact with PKC prior to their phosphorylation [28,29]; receptors for activated C-kinase (RACKs) proposed to bind at site(s) distinct from the substrate-binding pocket of the kinase [30,31]; PICK-1 (proteins that interact with C-kinase), a protein cloned in two-hybrid screens that interacts with the C-terminal tail of PKCα [32,33]; and GAP43, which binds PKCδ in neurons [34]. However, AKAP79 represents a distinct class of PKC-targeting protein, as the anchoring protein inhibits kinase activity as it binds to the catalytic core of the enzyme. In fact, all

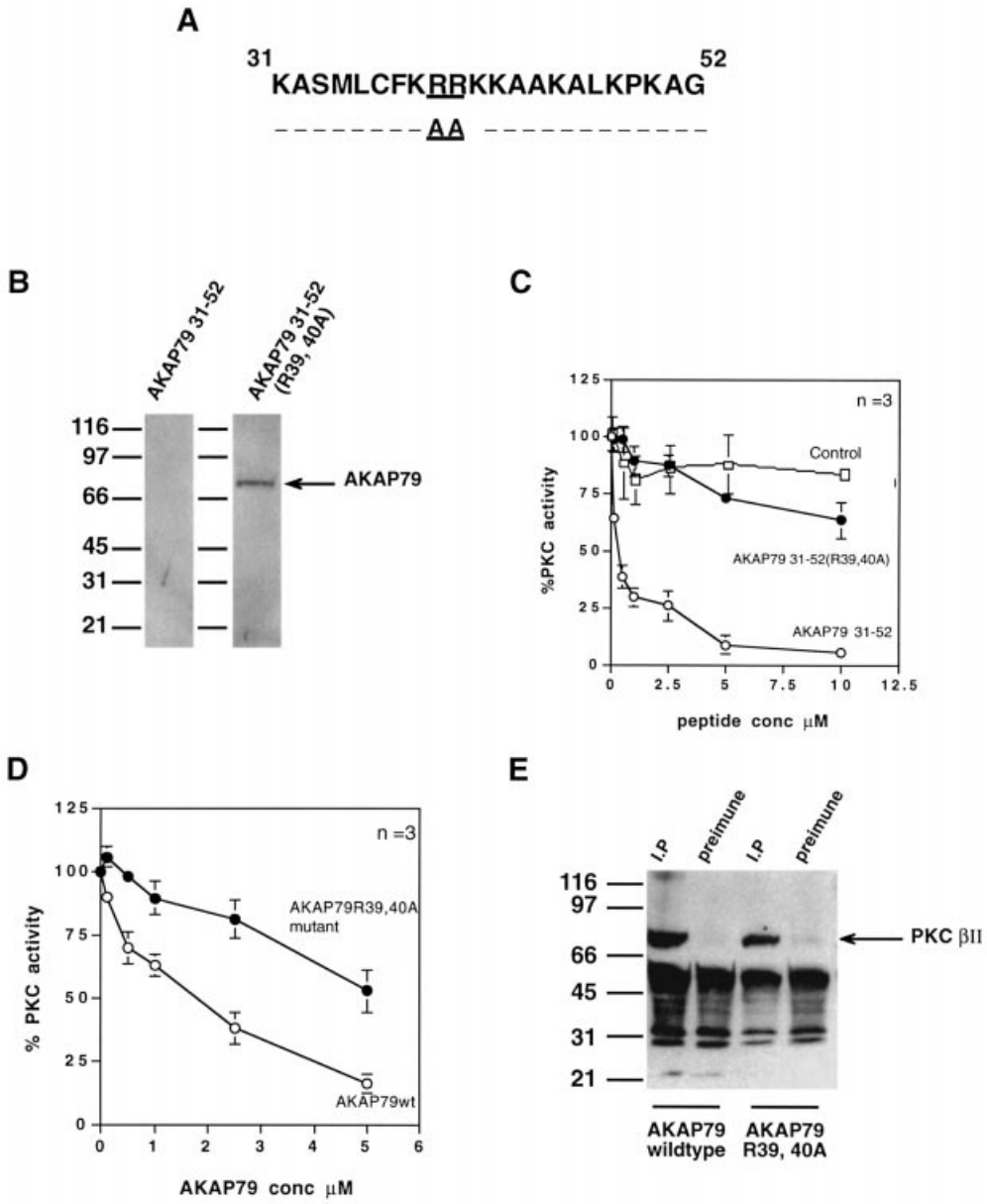

Figure 8 Arginines 39 and 40 in AKAP79 contribute to inhibition of PKCβII activity

(**A**) Amino acid sequences of the AKAP79(31–52) peptide and the modified AKAP79(31–52) (R39,40A) peptide. (**B**) AKAP79 was blotted and PKCβII overlays performed in the presence of 2.0 μM AKAP79(31–52) peptide (left panel) and 2.0 μM AKAP79(31–52) (R39,40A) (right panel). (**C**) Concentration response of PKCβII activity assayed in the presence of AKAP79(31–52) (○), AKAP79(31–52) (R39,40A) (●) and control (□) peptides. Values shown are mean ± S.E.M. ($n = 3$). In order to test the role of both arginine residues within the context of the full-length protein or double mutant, AKAP79 (R39,40A) was constructed and purified as described in the Experimental section. (**D**) Concentration–response curve of PKCβII activity assayed in the presence of AKAP79 wild-type protein (○) and AKAP79 (R39,40A) mutant protein (●). Values shown are mean ± S.E.M. ($n = 3$). (**E**) AKAP79 wild-type protein (2 μg) or AKAP79 (R39,40A) mutant protein (2 μg) in the presence of soluble Sf21 cell lysate (100 μg) expressing PKCβII were incubated for 1 h at 4 °C. Proteins were immunoprecipitated with a polyclonal antibody to AKAP79 918I (I. P.) or preimmune serum as described in the Experimental section. Precipitated proteins were immunoblotted and probed with a monoclonal antibody to PKCα/β. Shown is a representative of at least three separate experiments. Molecular-mass markers are indicated in kDa.

of the PKC isoforms tested were able to bind AKAP79 *in vitro* and inside cells, in accordance with PKCs and AKAP79 being bound to membranes, whereas RACKs and PICK-1 have been reported to be isoform-selective [30,32]. Unlike the other PKC-targeting proteins, AKAP79 localizes three signalling enzymes, PKA, calcineurin and PKC, to postsynaptic membranes [11,12,35]. Association with the anchoring protein forms a signalling scaffold that optimally positions each enzyme just below the postsynaptic membrane where it can respond individually to activation signals such as the influx of calcium or the generation of the second messengers, cAMP and DG [13]. Thus AKAP79 may represent a point of convergence for Ca^{2+}–calmodulin- and DG-signalling pathways that regulate PKC-phosphorylation events at the postsynaptic membrane.

Another issue addressed in this study relates to the site of AKAP79 interaction with the kinase. PKC is maintained in a dormant state through intrasteric interactions between a pseudosubstrate region in the regulatory domain and the sub-

strate-binding cleft of the kinase catalytic core [27]. Since PKC is inactive when bound to AKAP79, it seemed likely that the active site of the kinase would participate in protein–protein interactions with the anchoring protein, particularly as the PKCβII catalytic core binds and is inhibited by AKAP79. However, point mutations in the ATP-binding site or the activation loop of PKC that render the kinase inactive did not prevent AKAP79 binding. These results suggest two possibilities: an active kinase conformation is not required for interaction with AKAP79, and/or that there are multiple sites of contact on the kinase core for association with the anchoring protein. In support of the latter hypothesis, we have evidence that the AKAP79 (R39,40A) mutant still binds the PKC catalytic core but inhibits the kinase to a lesser extent (M. C. Faux and J. D. Scott, unpublished work). One site of contact may include the substrate-binding groove on the lower lobe of the kinase catalytic core, as we have shown that the mechanism of AKAP79(31–52) peptide inhibition demonstrates strong elements of competitive inhibition [10]. Moreover, proteolysis experiments imply that binding of the anchoring protein alters the conformation of PKC in a way that exposes the auto-inhibitory pseudosubstrate. On the basis of these data it is reasonable to postulate that PKC adopts an open conformation [27] when associated with AKAP79. Interestingly, our findings also indicate that there is no apparent involvement of the PKC activator DG or phospholipid cofactors for interaction with the anchoring protein. This indicates further that the kinase is not required to undergo full activation in order to associate with the anchoring protein, and places more emphasis on the role of protein–protein interactions for PKC–AKAP79 interaction. However, lipids do bind other sites on AKAP79, as recent targeting studies have identified regions of basic amino acids on the anchoring protein that facilitate association with the plasma membrane through interaction with acidic phospholipids [11].

The molecular basis for pseudosubstrate inhibition was developed from the concept that regulatory sequences mimicking substrate-recognition motifs are able to compete for binding to the active site of a kinase [36]. Basic residues represent key determinants in PKC–pseudosubstrate interactions, and molecular modelling of the PKC catalytic core reveals a cluster of acidic residues within the substrate-binding groove that may form a reciprocal binding surface [27]. As with the pseudosubstrate, the principal PKC-binding site on AKAP79 (residues 31–52) is highly basic. Another parallel with pseudosubstrate-like interactions is provided by evidence that the AKAP79(31–52) peptide inhibits PKC activity and results in exposure of Arg-19 in the pseudosubstrate to proteolysis. These observations prompted us to explore the contribution of two arginine residues in AKAP79 as determinants for PKC binding. Evidence that the modified AKAP79(31–52) (R39,40A) peptide was neither able to block AKAP79–PKC binding nor inhibit kinase activity supported the notion that these basic residues within the corresponding region on the anchoring protein interact with PKC. However, additional binding determinants are present within this region, as mutation of arginines 39 and 40 in the full-length AKAP79 protein produces less-pronounced effects on PKC inhibition and the mutated protein retained some ability to bind PKC. It is likely that any additional binding determinants must reside within the first 75 residues of the anchoring protein, as we have shown previously that this region alone is sufficient to bind and completely inhibit PKC activity [10].

The proposed function for AKAP79 in neurons is to provide a scaffold for PKA, PKC and calcineurin at postsynaptic sites [10]. Targeting of such a multi-enzyme signalling complex would allow local control of phosphorylation events mediated by second-messenger signals such as the generation of cAMP, calcium and DG. Activation of PKA or PKC could facilitate phosphorylation of synaptic substrates such as ion channels and neurotransmitter receptors [37,38]. AKAP79 concentrates these enzymes at their sites of action, as demonstrated by functional studies showing that disruption of PKA anchoring with peptide inhibitors alters the modulation of ionotropic glutamate receptors [37]. Subsequent studies have used AKAP79 or AKAP18 as vectors that target PKA to the membrane, thereby favouring cAMP-dependent stimulation of substrates such as the L-type Ca^{2+} channel [19,39]. Whereas the functional consequences of PKC interaction with AKAP79 are yet to be determined, it is interesting to note that our immunochemical data imply that certain PKC isoforms appear to be most prevalent in the cell bodies (Figure 6). Since PKC phosphorylation augments the ion flow through *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptors, which are concentrated in soma, it is tempting to speculate that PKC anchoring provides a mechanism for facilitating phosphorylation at these sites [40,41]. In addition, the dendritic location of PKCϵ, and to a lesser extent PKCι, is consistent with a proportion of these PKC isoforms being anchored at postsynaptic sites (Figures 6E and 6H). Another proposed function for PKC interaction with AKAP79 is phosphorylation of the anchoring protein itself. AKAP79 is a substrate for PKC *in vivo*, and phosphorylation by PKC regulates targeting of the anchoring protein to phospholipid membranes [11]. Thus it is plausible that AKAP79 binds and inhibits PKC in order to maintain a pool of kinase at the optimum site for phosphorylation of the anchoring protein in response to the generation of second messengers. This would involve the concerted and ordered action of two signals: Ca^{2+}–calmodulin, which releases the inhibited kinase from contact with the anchoring protein [14], and the PKC activator DG. Future studies are planned to assess the contribution of AKAP79 in the functional coupling of PKC with selected synaptic substrates.

We thank Dariano Fabbro (Novartis, Palo Alto, CA, U.S.A.) for a generous gift of baculoviral constructs of PKCα, δ, ϵ, and ζ, and Lisa Keranan for construction of the PKC K371R mutant. We also thank Yvonne Lai (Icos, Seattle, WA, U.S.A.) for providing anti-AKAP79 (918I) and anti-AKAP150 (4361J) antibodies as well as baculoviral constructs for PKCθ. We are especially grateful to Ann Westphal for preparation of primary cultures of rat hippocampal neurons for immunofluorescence, and our colleagues at the Vollum Institute for critical evaluation of the manuscript. This work was supported by National Institutes of Health grants GM48231 to J.D.S. and GM43154 to A. C. N.

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Received 30 April 1999/27 July 1999; accepted 17 August 1999