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Regulation of the AKAP79-Protein Kinase C Interaction by Ca^{2+}/Calmodulin*

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The A kinase-anchoring protein AKAP79 coordinates the location of the cAMP-dependent protein kinase (protein kinase A), calcineurin, and protein kinase C (PKC) at the postsynaptic densities in neurons. Individual enzymes in the AKAP79 signaling complex are regulated by distinct second messenger signals; however, both PKC and calcineurin are inhibited when associated with the anchoring protein, suggesting that additional regulatory signals must be required to release active enzyme. This report focuses on the regulation of AKAP79-PKC interaction by calmodulin. AKAP79 binds calmodulin with high affinity (K_D of 28 ± 4 nM (n = 3)) in a Ca^{2+}-dependent manner. Immunofluorescence staining shows that both proteins exhibit overlapping staining patterns in cultured hippocampal neurons. Calmodulin reversed the inhibition of PKCβII by the AKAP79(31–52) peptide and reduced inhibition by the full-length AKAP79 protein. The effect of calmodulin on inhibition of a constitutively active PKC fragment by the AKAP79(31–52) peptide was shown to be partially dependent on Ca^{2+}. Ca^{2+}/calmodulin reduced PKC coimmunoprecipitated with AKAP79 and resulted in a 2.6 ± 0.5-fold (n = 6) increase in PKC activity in a preparation of postsynaptic densities. Collectively, these findings suggest that Ca^{2+}/calmodulin competes with PKC for binding to AKAP79, releasing the inhibited kinase from its association with the anchoring protein.

Protein phosphorylation of intracellular substrates by kinases and phosphatases controls many aspects of cellular function (1). As the individual components of many signaling pathways have been identified, it has become apparent that the regulation of phosphorylation events is achieved at many levels. Although soluble second messengers control the activity state of kinases and phosphatases, other factors influence where and when these enzymes have access to their substrates. Localization of kinases and phosphatases adds a measure of selectivity to their action as it restricts which phosphorylation events occur in response to a particular stimulus. Consequently, several prominent classes of serine/threonine protein kinases and phosphatases are compartmentalized through interactions with anchoring or targeting proteins (2–4). For example, protein phosphatase 1 associates with targeting subunits that localize the catalytic subunit and adapt catalytic activity to preferentially dephosphorylate certain substrates (5, 6).

An emerging family of proteins called AKAPs[1] (A Kinase Anchoring Proteins) binds to the regulatory subunit of PKA, localizing the kinase to particular cellular locations, primed for activation by cAMP (for review, see Ref. 7). Some AKAPs bind more than one signaling enzyme. For example, AKAP79 not only associates with PKA but also binds protein phosphatase 2B, calcineurin, and protein kinase C, whereas another anchoring protein, gravin, binds PKA and PKC (8–11). These multivalent binding proteins serve as scaffolds for multienzyme signaling complexes. We have proposed that these signaling scaffolds preferentially control the phosphorylation of selected substrates such as ion channels and cytoskeletal components through integration of signals from distinct second messengers such as Ca^{2+} and cAMP (12). However, regulation of these signaling complexes is not fully understood. Although cAMP releases the catalytic subunit of PKA from AKAP79, it is evident that additional regulatory mechanisms must be involved to release inhibited calcineurin and PKC from their association with the anchoring protein.

In this report we have focused on the regulation of PKC targeting by AKAP79. Although the PKC family of at least 11 phospholipid-dependent enzymes is activated in response to the generation of diacylglycerol and in some cases Ca^{2+}, most of the isoforms have nearly identical substrate specificities (13, 14). Differential localization may contribute to the specificity of PKC action, as a combination of subcellular fractionation and immunocytochemical studies have demonstrated that certain PKC isoforms are found in different cellular compartments (15, 16). Although localization of PKC primarily involves protein-lipid interactions, it is now apparent that PKC-targeting proteins are also important in directing the location of the kinase to particular parts of the cell (14). There are several classes of PKC-targeting proteins: substrate-binding proteins that bind PKC in the presence of phosphatidylserine (17); receptors for activated C-kinase which are not necessarily substrates for PKC and bind at site(s) distinct from the substrate binding pocket of the kinase (18); and proteins that interact with C-kinase which have been cloned in two hybrid screens using the catalytic core of the kinase as bait (19). We have recently demonstrated that PKC binds AKAP79 in what appears to represent a unique class of PKC-binding proteins. PKC binds AKAP79 in a phosphatidylserine-dependent manner through an amino-terminal basic and hydrophobic sequence and is inhibited by the anchoring protein (10). We now show that Ca^{2+}/calmodulin antagonizes this interaction presumably by competing for association with this region on AKAP79. This

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[1] The abbreviations used are: AKAP(s), A kinase anchoring protein(s); PKA, cAMP-dependent protein kinase (protein kinase A); PKC, protein kinase C; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PSD, postsynaptic density; MARCKS, myristolated alanine-rich C kinase substrate.

provides a Ca^{2+}-dependent regulatory mechanism for releasing the inhibited kinase from its association with the anchoring protein.

EXPERIMENTAL PROCEDURES

Calmodulin-Agarose Affinity Purification, SDS-Polyacrylamide Gel Electrophoresis, and Immunoblotting—Recombinant AKAP79 protein was expressed in *Escherichia coli* as described (8). AKAP79 (5 μg) was incubated with calmodulin-agarose (Sigma) (20 μl of packed beads) in Buffer A (20 mM Tris, pH 7.0, 1 mM imidazole, 1 mM magnesium acetate, 0.05% w/v Nonidet P-40, 15 mM β-mercaptoethanol, 1 mM benzamidine, 2 μg/ml pepstatin, 2 μg/ml leupeptin, and 100 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride) containing 0.2 mM $CaCl_2$ for 2 h at 4 °C. The resin was washed five times with Buffer A containing 5 M NaCl and 0.2 mM $CaCl_2$ and then three times with Buffer A containing 0.2 mM $CaCl_2$. AKAP79 was eluted following incubation with 2 mM EGTA for 1 h at 4 °C. The eluted proteins were boiled for 5 min in SDS-sample preparation buffer, separated by SDS-polyacrylamide gel electrophoresis (10% gel) (20), and immunoblotted (21). AKAP79 was detected with affinity-purified rabbit polyclonal antibody 918I. This procedure was repeated in the presence of Buffer A containing 0.2 mM EGTA, and proteins were eluted with Buffer A containing 5 mM $CaCl_2$.

Surface Plasmon Resonance Measurements—Biotinylated calmodulin (Life Technologies, Inc.) was coupled to a carboxymethyl dextran IAsys cuvette (Affinity Sensors) via NeutrAvidin™ (Pierce) using standard 1-ethyl-3(3-dimethylaminopropyl) carbodiimide, *N*-hydroxysuccinimide coupling chemistry (22). Briefly, the cuvette was activated by treating with 0.4 M 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (Pierce), 0.1 M *N*-hydroxysuccinimide (Pierce) for 15 min and washed extensively with PBST (PBS containing 1 mM Ca^{2+} and 1 mM Mg^{2+} (Life Technologies, Inc.) + 0.05% Tween 20 (Amersham)). Excess NeutrAvidin was coupled to the activated cuvette in 10 mM sodium acetate buffer, pH 4.5, for 30 min at room temperature. Uncoupled protein was washed away with PBST and free amines blocked with 1 M ethanolamine (Affinity Sensors). After washing extensively with PBST, 1 μM AKAP79 recombinant protein was added to check that it did not bind nonspecifically to the cuvette surface. After washing with PBST, 2 μg of biotinylated calmodulin was then coupled to the cuvette via the NeutrAvidin. The calmodulin cuvette was washed with 5 M NaCl and PBST and a stable base line was established for 10 min before data collection. All binding experiments were performed with AKAP79 recombinant protein over a range of concentrations from 5 to 100 nM in volumes of 200 μl in PBST. The binding surface was regenerated with short (1 s) pulses of 6 M guanidinium HCl with no decrease in extent measurements for the duration of the experiments which were completed within 1 day. Data were collected over 3-s intervals and were analyzed using the Fastfit™ software which was provided with the IAsys instrument. The K_D value was confirmed with analysis of extent data plotted *versus* AKAP79 concentration which yielded an equilibrium constant that was in good agreement with the K_D obtained from the rate data.

Immunocytochemistry—Cultured neonatal rat hippocampal neurons grown on coverslips were rinsed with PBS, fixed in 3.7% formaldehyde (5 min), and extracted in −20 °C absolute acetone for 1 min. Cells were rehydrated in PBS containing 0.2% BSA for 1 h and then incubated with a mixture containing affinity-purified rabbit anti-AKAP79 antibody 2503 at 1.5 μg/ml and mouse anti-calmodulin antibody (Upstate Biotechnology, Inc.) at 1.2 μg/ml in PBS containing 0.2% BSA for 1 h. Coverslips were washed three times with PBS containing 0.2% BSA and incubated with a mixture of fluorescein isothiocyanate-conjugated anti-rabbit (1:500) (Molecular Probes) and Texas Red-conjugated anti-mouse (1:250) (Molecular Probes) secondary antibodies in PBS containing 0.2% BSA for 1 h. The coverslips were then washed three times in PBS containing 0.2% BSA and mounted with Slow Fade Antifade (Molecular Probes). Cells were analyzed with a Leitz Fluovert FU confocal photomicroscope with a Leitz 40/1.6 N.A. lens. Specific staining was not detected in control experiments with secondary antibody alone.

Protein Kinase Assays—PKCβII, from a baculovirus expression system, was purified as described (23) and was a generous gift of Dr. Alexandra Newton (University of California, San Diego). PKC activity was assayed as described (24) in a 40-μl reaction containing 40 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 μM [γ-^{32}P]ATP (500 cpm/pmol) with epidermal growth factor receptor peptide (VRKRTLPRL) or PKC(19–31S25) peptide (RFARKGSLRQLNV) as substrates at 30 °C. Assays were performed with or without activators: in the presence of either 300 μM $CaCl_2$ and Triton X-100 (0.1% w/v) micelles containing 20 mol % phosphatidylserine (Avanti) and 5 mol % 1,2-dioleoylglycerol (Avanti), or 2 mM EGTA and Triton X-100 (0.1% w/v), or in the presence of 300 μM $CaCl_2$ and phosphatidylserine (20 μg/ml), or 2 mM EGTA. Triton X-100:phosphatidylserine:diacylglycerol-mixed micelles were prepared as described (25). PKCβII was diluted in 20 mM Tris, pH 7.9, 1 mg/ml BSA, and 1 mM dithiothreitol. Time course experiments were performed over 15 min with 10 μM AKAP79 peptide plus or minus 10 μM calmodulin. Purified bovine calmodulin was a generous gift of Dr. Roger Colbran (Vanderbilt University). Dose-response curves were generated over an inhibitor concentration range of 0.1–10 μM AKAP79 protein and AKAP79(31–52) peptide in the presence or absence of 10 μM calmodulin. PKC activity was assayed in a preparation of rat forebrain postsynaptic densities, a generous gift of Dr. Roger Colbran (26), following incubation with or without calmodulin for 15 min. Assays were performed in the presence of PKC activators: 300 μM $CaCl_2$ and Triton X-100 (0.1% w/v) micelles containing 20 mol % phosphatidylserine and 5 mol % 1,2-dioleoylglycerol and kinase inhibitors PKI(5–25) and KN62. The assays were performed in the presence and absence of PKC(19–36) pseudosubstrate peptide (1 μM).

Limited Trypsin Digestion—PKCβII (30 nmol) was digested with trypsin (300 ng/ml) (Sigma) in a 15-μl reaction in 20 mM HEPES, pH 7.0, and 1 mM dithiothreitol for 8 min at 30 °C as described (25). The reaction was terminated with excess soybean trypsin inhibitor (Sigma) and the digested material placed on ice. Proteolytic products were then assayed for PKC activity in the presence of $CaCl_2$ (300 μM) or EGTA (2 mM) plus or minus calmodulin over a range of concentrations of 0.1 to 10 μM AKAP79(31–52) peptide.

Immunoprecipitation of AKAP79—A partially purified preparation of PKC was prepared from rabbit brain as described (27). AKAP79 protein (10 μg) was incubated with rabbit brain PKC (2 μg) in the presence or absence of bovine calmodulin in hypotonic buffer (10 mM HEPES, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, Nonidet P-40 (0.5% w/v), 1 mM benzamidine, 2 μg/ml pepstatin, 2 μg/ml leupeptin, and 100 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride) containing 1 mM $CaCl_2$ for 1 h at 4 °C. Samples were then incubated with either affinity-purified anti-AKAP79 918I antibodies (4 μg) or preimmune IgG (4 μg) at 4 °C for 18 h. Immune complexes were isolated by the addition of 100 μl of 10% v/v protein A-Sepharose CL-4B (Sigma) which had been equilibrated in hypotonic buffer containing 1 mM $CaCl_2$. Following incubation at 4 °C for 60 min, the beads were washed three times with hypotonic buffer containing 1 mM $CaCl_2$ and 1 M NaCl and three times with hypotonic buffer containing 1 mM $CaCl_2$. The immunoprecipitation experiment was repeated in the presence of hypotonic buffer containing 0.5 mM EGTA. For immunoblot analysis, precipitated proteins were eluted by boiling the washed beads in SDS-sample preparation buffer for 5 min and separated by SDS-polyacrylamide gel electrophoresis on a 10% denaturing gel. Proteins were transferred to nitrocellulose and analyzed by Western blot with an antibody to PKCα/β (Transduction Laboratories). For measuring PKC activity associated with the beads, the washed beads were resuspended in 60 μl of hypotonic buffer containing 1 mM $CaCl_2$ and assayed for PKC activity in duplicate. PKC activity was expressed as fold increase over PKC activity associated with preimmune complexes.

RESULTS

AKAP79 Is a Ca^{2+}/Calmodulin-binding Protein—We have shown previously that PKC associates with AKAP79, and the principal site of contact lies between residues 31 and 52 on the anchoring protein (10) (Fig. 1*A*). This stretch of alternating basic and hydrophobic residues also resembles a calmodulin-binding domain (28). As previous studies have shown that AKAP79 and the bovine homolog AKAP75 are calmodulin-binding proteins (8, 29), we wanted to determine if binding was Ca^{2+}-dependent. Immunoblot analysis using antibodies to AKAP79 demonstrates that AKAP79 binds calmodulin-agarose in the presence of 0.2 mM Ca^{2+}, and binding is abolished in the presence of 2 mM EGTA (Fig. 1*B*). This suggests that AKAP79 binds calmodulin in a Ca^{2+}-dependent manner.

To investigate further the interaction between AKAP79 and calmodulin *in vitro*, the binding affinity of recombinant AKAP79 to biotinylated calmodulin was measured by surface plasmon resonance (Fig. 2). Binding of AKAP79 to immobilized calmodulin in the presence of excess Ca^{2+} was measured over a range of AKAP79 concentrations (5–100 nM) (Fig. 2*A*). Analysis of the association rate data showed that the binding was biphasic, which may result from steric problems or may indi-

cate more than one site of binding. The association rate constant (k_a 203,029 ± 24,805 M^{-1} s^{-1}, n = 3) was obtained by plotting the measured k_{on} *versus* AKAP79 concentration (Fig. 2*B*). The dissociation rate constant (k_d 0.0055 ± 0.00043 s^{-1} n = 3) was obtained directly from dissociation experiments. These values were used to calculate a K_D of 28 ± 4 nM (n = 3) for the AKAP79-calmodulin interaction. The observed dissociation rate value obtained from the single phase fit of the data is in good agreement with the dissociation rate constant extrapolated from the plot of k_{on} *versus* AKAP79 concentration (Fig. 2*B*). The equilibrium constant of 28 nM is within the physiological concentration range for these proteins and is consistent with the idea that AKAP79 and calmodulin may associate in the cell.

Further evidence for the association of AKAP79 and calmodulin was provided by immunofluorescence staining of these proteins in cultured rat hippocampal neurons (Fig. 3). AKAP79 exhibits a distinct staining pattern concentrated at two subcellular locations: at the periphery of the cell body and in dendritic bundles within the neurite extensions (Fig. 3*A*). Calmodulin staining is similar, with staining at the periphery of the cell body and in the neurite extensions, although the calmodulin is more uniformly distributed throughout the neurites, and there is also staining in the nucleus of the cell (Fig. 3*B*). Double labeling experiments show considerable overlap of staining at the periphery of the cell and in neurite extensions, demonstrating that these proteins occupy the same focal plane (Fig. 3*C*). Collectively, these findings suggest that AKAP79 interacts with calmodulin, and it is feasible that this interaction occurs *in vivo*.

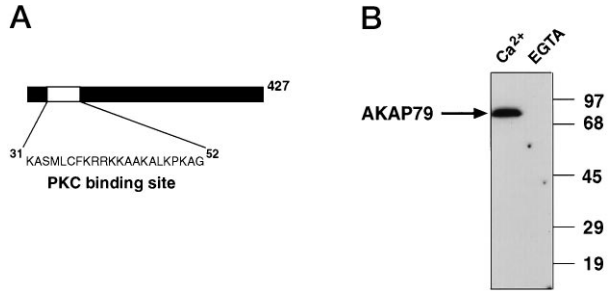

FIG. 1. **AKAP79 binds calmodulin in a Ca^{2+}-dependent manner.** *Panel A*, schematic representation of AKAP79 showing putative PKC binding site. The amino acid sequence for residues 31–52 is indicated in the single letter amino acid code. *Panel B*, AKAP79 (5 μg) was incubated with calmodulin-agarose (20 μl of packed beads) for 2 h at 4 °C in the presence of either 0.1 mM Ca^{2+} or 0.2 mM EGTA as indicated (described under "Experimental Procedures"). Proteins were eluted with 2 mM EGTA or 5 mM Ca^{2+}, respectively, analyzed by SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose for Western blot analysis. The calmodulin-agarose beads were eluted with SDS-sample preparation buffer and analyzed by Western blot. There was no AKAP79 detected when incubated in the presence of 0.2 mM EGTA. Immunoblots were probed with antibodies to AKAP79. Molecular mass markers are indicated in kDa.

Calmodulin Releases PKC from Inhibition by AKAP79—The region on AKAP79 which binds PKC resembles a calmodulin binding sequence (Fig. 1*A*). The idea that calmodulin and PKC may share common binding determinants suggested the intriguing possibility that Ca^{2+}/calmodulin may play a role in regulating the interaction of PKC with AKAP79. We therefore investigated the effect of Ca^{2+}/calmodulin on the inhibition of PKC activity by AKAP79. AKAP79 and a peptide encompassing residues 31–52 of the anchoring protein inhibit PKC activity (10). Addition of Ca^{2+}/calmodulin to the reaction releases PKC activity so that it is no longer inhibited by AKAP79 (Fig. 4). Fig. 4*A* shows a time course of PKC activity. PKC activity increases in a linear fashion over 15 min. In the presence of the AKAP79(31–52) peptide (10 μM), PKC activity is inhibited; however, addition of 10 μM calmodulin prevents inhibition of PKC activity (Fig. 4*A*). Since calmodulin alone does not affect the activity of PKC (data not shown), it is likely that calmodulin competes for binding to the AKAP79(31–52) peptide. Similarly, PKC activity is inhibited by AKAP79(31–52) peptide in a concentration-dependent manner; but when 10 μM calmodulin is added, the peptide no longer inhibits PKC activity (Fig. 4*B*). The relief of inhibition by AKAP79(31–52) in the presence of calmodulin is specific as inhibition by the pseudosubstrate PKC(19–36) peptide is not affected by calmodulin (data not shown). When AKAP79(31–52) peptide (100 μM) is present in excess over calmodulin (10 μM), PKC activity is once more inhibited (not shown). These data suggest a potential mechanism of regulation whereby Ca^{2+}/calmodulin competes with PKC for binding to the AKAP79(31–52) peptide relieving the inhibition of PKC activity.

Calmodulin also affects the inhibition of PKC by the recombinant AKAP79 protein (Fig. 4*C*). Addition of 10 μM calmodulin causes a shift in the dose-response curve such that the inhibition is reduced. Interestingly, this effect is not as dramatic as that seen with the AKAP79(31–52) peptide, which is somewhat surprising given the high affinity interaction between AKAP79 and calmodulin. However, the binding studies with AKAP79 and calmodulin demonstrate biphasic association, suggesting the potential for binding at more than one site. Thus, calmodulin does not fully prevent inhibition under these conditions. This suggests that calmodulin partially relieves the inhibition

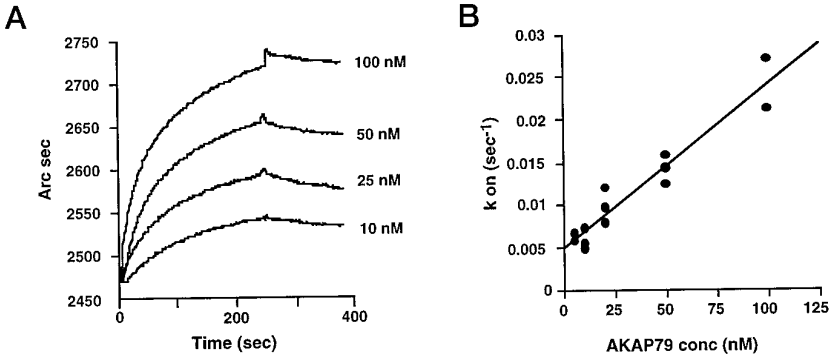

FIG. 2. **Affinity measurement of AKAP79 and calmodulin.** The binding affinity of recombinant AKAP79 and calmodulin was measured by surface plasmon resonance. Biotinylated calmodulin was immobilized on the surface of the IAsys (Fison) cuvette as described under "Experimental Procedures." The immobilized calmodulin was incubated with AKAP79 over a range of concentrations (5–100 nM). *Panel A*, real time binding measurements showing the binding profiles for selected concentrations of AKAP79. Binding was measured as a function of plasmon resonance extent (*Arc sec*). *Panel B*, measured on rates (s^{-1}) plotted *versus* concentration of AKAP79. Measurements were completed in triplicate. Shown is a representative plot from three experiments.

of PKC by the AKAP by competing with PKC for binding to AKAP79. Interestingly, the anchoring protein inhibits PKC activity more potently in the presence of phosphatidylserine, and this inhibition is relieved slightly with diacylglycerol/phosphatidylserine micelles (Fig. 4*D*). Inhibition of PKC by the anchoring protein is relieved to a greater extent in the presence of Ca^{2+}/calmodulin with diacylglycerol/phosphatidylserine micelles relative to phosphatidylserine (Fig. 4*D*). This suggests that Ca^{2+}/calmodulin as well as the second messenger diacylglycerol may coordinate to relieve PKC from inhibition by AKAP79.

Effect of Calmodulin on Ca^{2+}-independent PKC Activity—As calmodulin binding to AKAP79 requires Ca^{2+}, we wished to investigate whether relief of PKC inhibition by the AKAP79(31–52) peptide was Ca^{2+}-dependent. Since PKCβII activity also requires Ca^{2+}, it was necessary to generate a Ca^{2+}-independent form of PKC. Limited trypsin digestion of PKC was used to liberate the constitutively active, Ca^{2+}-independent catalytic core of the enzyme (Fig. 5*A*). Inhibition of Ca^{2+}-independent PKC activity by the AKAP79(31–52) peptide was the same as the intact kinase either in the presence or absence of Ca^{2+} (Fig. 5*B*). This suggests that the peptide binds at the catalytic core of the kinase, which is consistent with previous kinetic studies on the mechanism of inhibition by the AKAP79(31–52) peptide (10). Addition of Ca^{2+}/calmodulin (10 μM) resulted in complete relief of inhibition of the constitutively active PKC (Fig. 5*B*). Addition of calmodulin in the presence of 2 mM EGTA reduced the inhibition, suggesting that the effect of calmodulin on PKC activity in the presence of the AKAP(31–52) peptide is partially dependent on Ca^{2+}.

Ca^{2+}/Calmodulin Reduces PKC Coimmunoprecipitated with AKAP79—Having shown that calmodulin reverses the inhibition of PKC activity by the AKAP79(31–52) peptide and reduces the inhibition of PKC activity by the full-length protein, we wanted to look more directly at the effect of calmodulin on PKC binding to AKAP79. Coimmunoprecipitation experiments were performed with an antibody to AKAP79 where recombinant AKAP79 was incubated with PKC in the presence or absence of calmodulin (Fig. 6). Immunoblot analysis demonstrates PKC coimmunoprecipitated with AKAP79 in the presence of either Ca^{2+} or EGTA (Fig. 6*A*, *lanes 1* and *5*). Preimmune serum was used as a control (Fig. 6*A*, *lanes 2*, *4*, *6*, and *8*). When Ca^{2+}/calmodulin was present, PKC was no longer coimmunoprecipitated (Fig. 6*A*, *lane 3*), whereas in the presence of EGTA, calmodulin did not prevent PKC coimmunoprecipitation (Fig. 6*A*, *lane 7*). Thus calmodulin competes with PKC for binding to AKAP79 in a Ca^{2+}-dependent manner. To obtain a more quantitative evaluation, complementary experiments measured PKC activity in immunoprecipitates. Ca^{2+}/calmodulin markedly reduced PKC activity coimmunoprecipitated with AKAP79 (Fig. 6*B*). This effect was Ca^{2+}-dependent as the presence of EGTA/calmodulin did not reduce PKC activity. These findings support the idea that Ca^{2+}/calmodulin regulates the

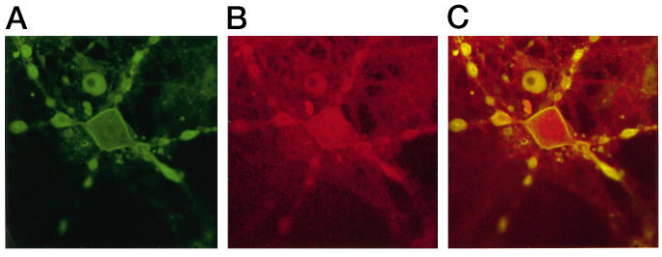

FIG. 3. **Subcellular distribution of AKAP79 and calmodulin in neurons.** Neonatal rat hippocampal neurons were fixed with 3.7% formaldehyde and permeabilized with 100% acetone at −20 °C for 1 min. Cells were incubated with antibodies to AKAP79 (affinity-purified rabbit polyclonal 2503) (*panel A*) and calmodulin (mouse monoclonal Upstate Biotechnology) (*panel B*) and detected with fluorescein isothiocyanate-conjugated anti-rabbit (1:500) or Texas red-conjugated anti-mouse (1:250) secondary antibodies, respectively. Images were analyzed by confocal microscopy as described under "Experimental Procedures." Double label immunofluorescence of AKAP79 and calmodulin was analyzed in the same focal plane (*panel C*). Fluorescence detection was by a Leitz Fluovert-FU confocal photomicroscope.

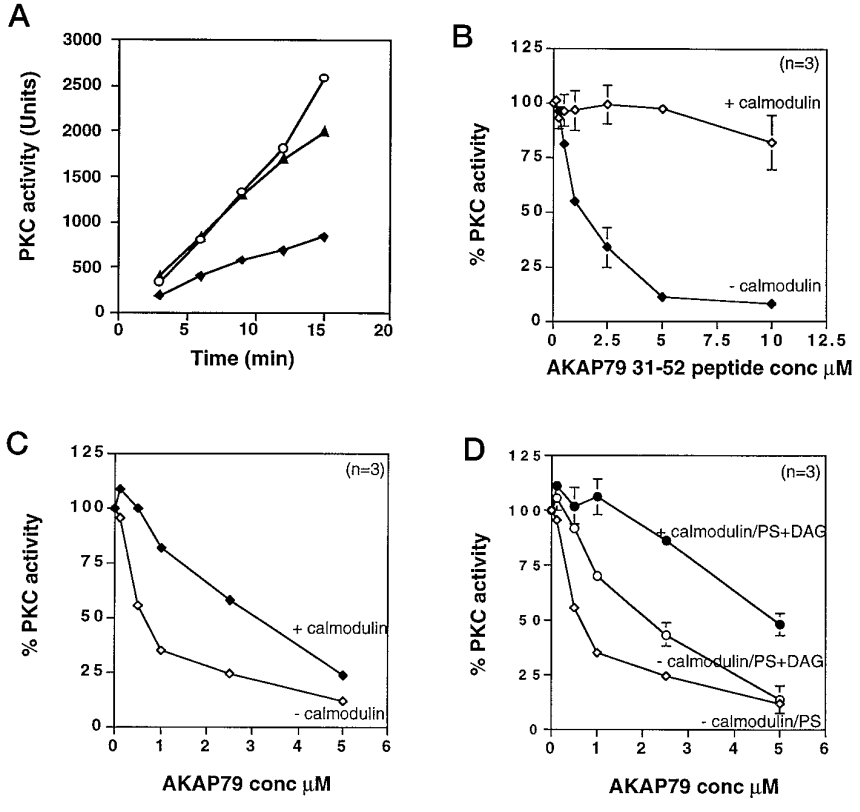

FIG. 4. **Calmodulin antagonizes AKAP79-PKC interaction.** *Panel A*, time course of PKCβII activity alone (*open circles*) or in the presence of 10 μM AKAP79(31–52) peptide (*filled diamonds*) and 10 μM AKAP79(31–52) peptide plus 10 μM calmodulin (*filled triangles*). Shown is a representative plot ($n = 3$). PKC activity is represented as units (μmol/mg). *Panel B*, dose-response curve of PKCβII activity in the presence of AKAP79(31–52) peptide (*filled symbols*) and AKAP79(31–52) peptide plus 10 μM calmodulin (*open symbols*). Values shown are mean ± S.E. ($n = 3$). *Panel C*, dose-response curve of PKCβII activity assayed in the presence of Ca^{2+} and phosphatidylserine, in the presence of AKAP79 recombinant protein (*open symbols*), and AKAP79 protein plus 10 μM calmodulin (*filled symbols*). Values shown are mean ± S.E. ($n = 3$). *Panel D*, dose-response curve of PKCβII activity assayed in the presence of Ca^{2+} and phosphatidylserine, in the presence of AKAP79 protein (*open diamonds*), and PKCβII activity assayed in the presence of Ca^{2+} and diacylglycerol/phosphatidylserine micelles, in the presence of AKAP79 recombinant protein (*open circles*), and AKAP79 protein plus 10 μM calmodulin (*filled circles*) as indicated. Values shown are mean ± S.E. ($n = 3$). PKC activity was measured as described under "Experimental Procedures."

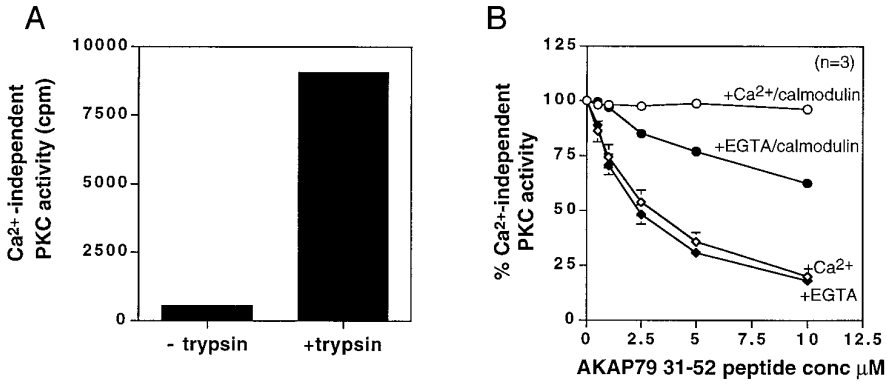

FIG. 5. **Release of inhibition by AKAP79(31–52) peptide by calmodulin is partially dependent on Ca^{2+}.** PKC (30 nmol) was digested with trypsin (300 ng/ml) to generate a constitutively active Ca^{2+}-independent enzyme as described under "Experimental Procedures." *Panel A*, Ca^{2+}-independent PKC activity following incubation of PKC either in the presence or absence of trypsin as indicated. *Panel B*, dose-response curve of Ca^{2+}-independent PKCβII activity in the presence of AKAP79(31–52) peptide in the presence of Ca^{2+} (*filled diamonds*), EGTA (*open diamonds*), Ca^{2+} plus calmodulin (*open circles*), and EGTA plus calmodulin (*filled circles*). Values shown are mean ± S.E. (n = 3). PKC activity was assayed as described under "Experimental Procedures."

interaction between AKAP79 and PKC by displacing PKC from its association with AKAP79.

Calmodulin Releases PKC Activity from Postsynaptic Densities—On the basis of *in vitro* experiments, we developed a working hypothesis that Ca^{2+}/calmodulin competes with PKC for binding to AKAP79, thus releasing the kinase from its inhibition by the anchoring protein. This idea is represented schematically in Fig. 7*A*. To test this idea in a more physiological context, PKC activity was measured in a preparation of postsynaptic densities (PSDs) (Fig. 7*B*). We have shown previously that AKAP79 is enriched at the PSD (8). To ensure that we were selectively measuring PKC activity, assays were performed using the PKC(19–31S25) peptide as substrate in the presence of the kinase inhibitors, PKI and KN62, to block the activities of PKA and calmodulin kinase II, respectively. PKC activity under these conditions was determined as counts inhibited by the PKC pseudosubstrate inhibitor peptide PKC(19–36). The 2.6 ± 0.5-fold (n = 6) increase in PKC activity following incubation with calmodulin (Fig. 7*B*) is consistent with the idea that calmodulin releases the inhibited kinase from its binding to the anchoring protein. Collectively these findings support a potential role for Ca^{2+}/calmodulin in regulating the release of PKC from anchored sites at the PSD.

DISCUSSION

The anchoring protein AKAP79 coordinates the location of PKA, PKC, and calcineurin (8–10). Targeting of these three signaling enzymes to submembrane sites such as the PSDs of neurons would ensure that each enzyme is well placed to receive signals transduced across the postsynaptic membrane (Fig. 7*A*). Although recent mapping studies have provided information on the individual enzyme binding sites on AKAP79, less is known about the regulation of the signaling complex. Our previous studies have shown that all three enzymes are inactive when bound to the anchoring protein (8–10). Although each enzyme responds to distinct second messenger signals generated at the postsynaptic membrane, additional factors contribute to their release and activation. PKA is directly activated following stimulation by its second messenger signal, cAMP (9). In contrast, both PKC and calcineurin are inhibited by AKAP79 even in the presence of their respective activators. Although the signals that release calcineurin from its association with AKAP79 remain to be elucidated, we have found that Ca^{2+}/calmodulin may play a role in regulating the interaction between PKC and AKAP79 (Fig. 7*A*). This suggests the potential convergence of Ca^{2+}/calmodulin and PKC signaling pathways in the regulation of PKC phosphorylation events at the PSD (Fig. 7*A*).

In this report we have shown that Ca^{2+}/calmodulin binds AKAP79 with high affinity and influences PKC activity by releasing the kinase from inhibition by the anchoring protein. Our evidence that excess Ca^{2+}/calmodulin overcomes the inhibitory effect of the AKAP79(31–52) peptide on PKC implicates this region as a potential regulatory site on the anchoring protein. This sequence exhibits the hallmarks of a calmodulin binding site in that it is rich in basic and hydrophobic residues (28). This suggests that calmodulin and PKC share the same or overlapping binding determinants on AKAP79. As AKAP79 is eluted from calmodulin-agarose by EGTA this demonstrates that binding is calcium-dependent. Although we could also purify the AKAP on calmodulin-agarose from bovine brain (9), interpretation of this result is complicated as a proportion of the AKAP is likely to be present through its association with calcineurin which is also a calmodulin-binding protein. However, the nanomolar affinity constant (calculated by surface plasmon resonance) and immunocytochemical data are consistent with the idea that AKAP79 and calmodulin interact inside cells.

Kinase activity measurements suggest that calmodulin prevents inhibition of PKC activity by the AKAP79(31–52) peptide but only partially prevents inhibition by the full-length protein. Although this was surprising given the high affinity interaction between AKAP79 and calmodulin, this result may reflect the complex nature of these protein-protein interactions. Since the full-length protein inhibits PKC more potently than the AKAP79(31–52) peptide, we cannot exclude the possibility that there are additional sites of contact on the anchoring protein for PKC which are less affected by calmodulin. Furthermore, calmodulin may bind to more than one site on the anchoring protein. For example, there are three regions on AKAP79 which are rich in basic and hydrophobic residues (8). Indeed, the surface plasmon resonance binding measurements indicated a biphasic association of AKAP79 with calmodulin, suggesting that there may be more than one site of interaction. These factors may contribute to Ca^{2+}/calmodulin being less effective when the full-length anchoring protein was used. The partial relief of inhibition by the full-length AKAP79 protein in the presence of calmodulin may also reflect the activation state of PKC when bound to the anchoring protein. PKC is activated following recruitment to the plasma membrane in response to diacylglycerol and Ca^{2+} (for Ca^{2+}-dependent isoforms) (30, 31). The interaction of PKC with AKAP79 requires phosphatidylserine, which may adapt the kinase to be in a particular orien-

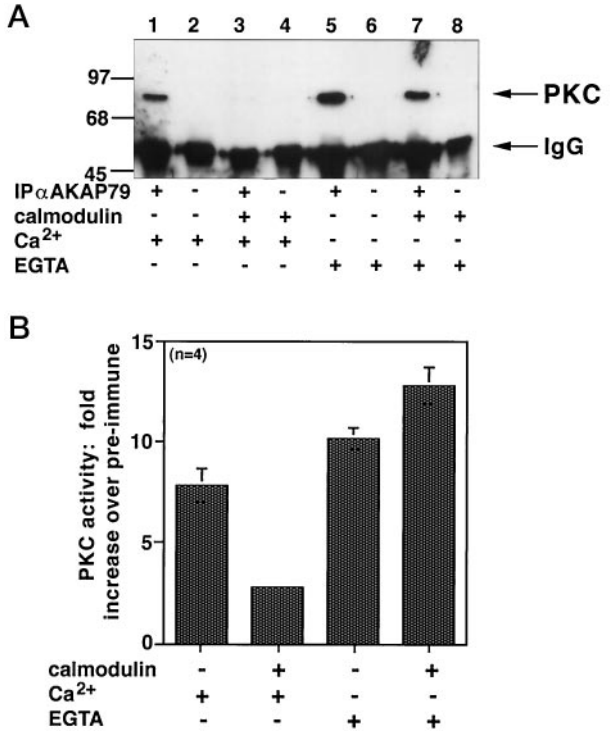

FIG. 6. **Ca^{2+}/calmodulin reduces PKC coimmunoprecipitated with AKAP79.** Recombinant AKAP79 protein (10 μg) was incubated with a partially pure preparation of PKC (2 μg) in the presence or absence of calmodulin (2 μM) in hypotonic buffer (see "Experimental Procedures"), containing either 1 mM Ca^{2+} or 0.5 mM EGTA for 1 h at 4 °C. Proteins were immunoprecipitated with an polyclonal antibody to AKAP79 918I or preimmune serum. *Panel A*, precipitated proteins were immunoblotted and probed with a monoclonal antibody to PKCα/β. Shown is a representative of at least three separate experiments. *Panel B*, following the final wash, the protein A-Sepharose beads were resuspended in 60 μl of hypotonic buffer and assayed for PKC activity. PKC activity is represented as fold increase over preimmune. Values shown are mean ± S.E. (n = 4). PKC activity was assayed as described under "Experimental Procedures."

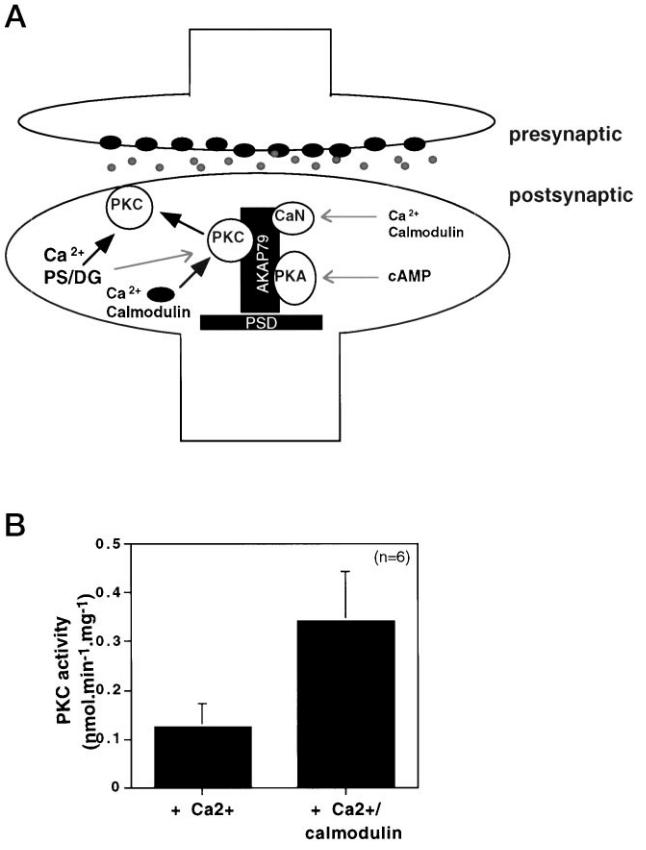

FIG. 7. **Calmodulin releases PKC activity from the postsynaptic density.** *Panel A*, schematic representation of working model that calmodulin releases PKC from its association with AKAP79 at the postsynaptic density by competing with PKC for binding to the anchoring protein. *Panel B*, rat forebrain postsynaptic densities were incubated with Ca^{2+} or Ca^{2+}/calmodulin and assayed for PKC activity. PKC activity was assayed in the presence of PKC activators (300 μM $CaCl_2$ and Triton X-100:phosphatidylserine:diacylglycerol-mixed micelles), kinase inhibitors PKI and KN62, using PKC(19–31, S25) peptide as substrate. PKC activity was taken as that inhibited by the PKC pseudosubstrate inhibitor, PKC(19–36) and assayed as described under "Experimental Procedures." Values shown are mean ± S.E. (n = 6).

tation for binding. Interestingly, the anchoring protein inhibits kinase activity more potently when phosphatidylserine is present, and this inhibition is partially reversed with diacylglycerol/phosphatidylserine micelles. Thus, it appears that PKC associates with AKAP79 by a mechanism that involves interactions with phosphatidylserine at the plasma membrane, but the presence of the second messenger diacylglycerol weakens the interaction. Although it is clear that binding of diacylglycerol is not sufficient to trigger PKC release, the combination of diacylglycerol and Ca^{2+}/calmodulin may synergize to release fully active PKC from its association with the anchoring protein. In support of this idea, inhibition of PKC by the full-length anchoring protein is relieved to a greater extent in the presence of Ca^{2+}/calmodulin with diacylglycerol/phosphatidylserine micelles relative to phosphatidylserine.

The integration of Ca^{2+}/calmodulin and PKC signaling pathways is reminiscent of regulation of the MARCKS protein (32, 33). MARCKS binds Ca^{2+}/calmodulin and cross-links actin. Calmodulin binding is dependent on Ca^{2+} and is prevented by PKC phosphorylation (34). Cross-linking of actin is disrupted by both phosphorylation and Ca^{2+}/calmodulin (35). There are several other examples of integration between PKC and calmodulin, such as neuromodulin (GAP43), neurogranin, and adducin. In each case phosphorylation by the kinase prevents association with calmodulin (36, 37). In contrast, calmodulin binding to AKAP79 does not appear to involve phosphorylation. The PKC binding site on AKAP79 does not contain a phosphorylation site, and phosphorylation does not affect PKC binding to the anchoring protein (10). It therefore seems unlikely that the interplay between Ca^{2+}/calmodulin and PKC is the same as for MARCKS. However, it is striking that there is such a precedence for convergence between Ca^{2+}/calmodulin and PKC signaling events.

Our coimmunoprecipitation experiments clearly show that Ca^{2+}/calmodulin reduces PKC coprecipitating with AKAP79. Presumably Ca^{2+}/calmodulin competes with PKC for binding to the anchoring protein. But does this regulation occur *in vivo*? The high concentration of calmodulin at the PSD (26) and our evidence for a high affinity interaction between Ca^{2+}/calmodulin and AKAP79 favor a model for Ca^{2+}/calmodulin-dependent antagonism of the AKAP79-PKC interaction. Consistent with this hypothesis are experiments measuring PKC activity from PSD preparations. The idea that the regulatory signal for release comes from a Ca^{2+}-dependent signaling event that does not directly activate the kinase is intriguing. This implies that Ca^{2+} acts at several places in the release and activation of anchored PKC. For example, calcium influx through channels at the postsynaptic membranes could dictate the release of anchored PKC through calmodulin, independently of kinase activation by lipid signaling, thereby introducing another level of organization for control of PKC phosphorylation events. Analogous to this idea is the finding that calmodulin binding to Ras-related GTP-binding proteins, Kir and Gem, inhibited

binding of GTP, leading the authors to suggest that calmodulin-binding motifs may represent an important module regulating protein-protein interactions in signal transduction pathways (38).

In conclusion, the data in this article present evidence for Ca^{2+}/calmodulin regulating the protein-protein interaction between PKC and AKAP79. These studies show that one component of the AKAP79 signaling complex, PKC, is regulated by the concerted action of two different second messengers: Ca^{2+}/calmodulin to release the kinase from the anchoring protein and Ca^{2+}/phospholipid, which is required to stimulate enzyme activity. This type of regulation represents another example of synergism between calmodulin and PKC signaling events. An intriguing aspect of this model is that Ca^{2+}/calmodulin is also involved in activating the calcineurin that is also a component of the AKAP79 complex. Future experiments will be designed to test this model inside the cell.

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