Dissection of Protein Kinase and Phosphatase Targeting Interactions

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Protein phosphorylation is a primary means of mediating signal transduction events that control cellular processes. Accordingly, the activities of protein kinases and phosphatases are highly regulated. One level of regulation is that the subcellular distribution of several kinases and phosphatases is restricted by association with targeting proteins or subunits. This mechanism promotes rapid and preferential modulation of specific targets within a defined microenvironment in response to diffusible second messengers. The type II cAMP-dependent protein kinase (PKA) is targeted by association of its regulatory subunit (RII) with A-kinase anchoring proteins (AKAPs). To date, 36 unique AKAPs have been identified. Each of these proteins contains a conserved amphipathic helix responsible for AKAP association with cellular structures. Disruption of PKA/AKAP interaction with peptides patterned after the amphipathic helix region blocks certain cAMP responses, including the modulation of glutamate receptor ion-channel activity in neurons and transcription of cAMP-responsive genes. Yeast two-hybrid screening methods have identified neuronal specific AKAP79-binding proteins including the β isoform of the phosphatase 2B, calcineurin. Biochemical and immunological studies have confirmed the two-hybrid results and identified additional members of this multienzyme signaling complex, including certain protein kinase C isoforms. These findings are consistent with colocalization of CaN, PKC, and type II PKA by AKAP79 and suggest a novel model for reversible phosphorylation in which the opposing kinase and phosphatase actions are colocalized in a signal transduction complex by association with a common anchor protein.

Introduction

Phosphorylation of protein substrates by kinases and phosphatases is a major process in the control of cellular function (Krebs and Beavo, 1979). Accordingly, the mechanisms involved in the regulation of kinase and phosphatase activity have been the subject of intense investigation since glycogen phosphorylase was first recognized to be regulated by phosphorylation (Fischer and Krebs, 1955; Krebs et al., 1959). One level of regulation involves control of enzyme activity, whereas the subcellular localization of kinases and phosphatases is restricted through interactions with targeting proteins (Kemp and Pearson, 1990; Faux and Scott, 1996*a*). The focus of this chapter will be to describe the use of synthetic peptides in identifying functional domains on a multivalent kinase and phosphatase targeting protein, the characterization of bioactive peptides in vitro, and the use of peptides as reagents to disrupt the localization or inhibition of enzyme activity in cells.

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The Targeting Hypothesis

Numerous studies have demonstrated that the regulation of reversible phosphorylation events is tightly controlled. In fact, a focus of this article is to highlight the importance of subcellular location as a means to control the actions of multifunctional protein kinases and phosphatases. Recent advances from a variety of laboratories indicate that the subcellular location of these enzymes is maintained by association with specific targeting proteins. For example, the cAMP dependent protein kinase (PKA), protein kinase C (PKC), and the Ca^{2+}/calmodulin-dependent protein kinase (CaM KII) are localized by specific binding proteins, and likewise, protein phosphatases 1, 2A, and 2B are positioned through association with "phosphatase targeting subunits" (Faux and Scott, 1996*a*). This has led to the formulation of a "targeting hypothesis" by Hubbard and Cohen which proposes that "targeting" subunits or proteins specify the location and catalytic and regulatory properties of protein kinases and phosphatases, and thereby play a key role in ensuring the fidelity of protein phosphorylation events (Hubbard and Cohen, 1993). The targeting subunit is defined as that part of a kinase or phosphatase which directs the catalytic subunit to a subcellular location (Fig. 1 *A*). A variation on this theme is the formation of kinase/phosphatase signaling complexes to modulate the phosphorylation state of specific target substrates (Fig. 1 *B*). Coordination of these complexes is achieved by multivalent targeting proteins which serve as platforms for the assembly of these signaling units (Faux and Scott, 1996*b*). The following sections describe the use of peptides as reagents to probe the function of kinase/phosphatase signaling complexes.

Compartmentalization of the cAMP-dependent Protein Kinase

The work of numerous investigators has shown that many hormone-stimulated signaling cascades emanate from transmembrane receptors at the plasma membrane and proceed through intermediary G-proteins to promote the stimulation of adenylyl cyclase (Wedegaertner et al., 1995). The net effect of this transduction system is the generation of the diffusible second messenger cAMP which binds and activates the cAMP-dependent protein kinase (PKA) (Walsh et al., 1968). The kinase is activated by the release of two catalytic subunits (C) from the regulatory (R) subunit-cAMP complex. An array of PKA isozymes are expressed in mammalian cells, and

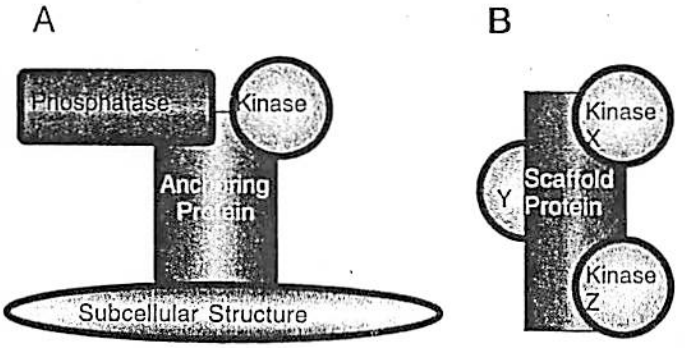

Figure 1. Localization of kinases and phosphatases through scaffold or anchoring proteins. (*A*) A schematic diagram depicting the topology of a prototypic kinase signaling scaffold and (*B*) an anchored kinase-phosphatase complex.

genes encoding three C subunits (Cα, Cβ and Cγ) and four R subunits (RIα, RIβ, RIIα and RIIβ) have been identified (reviewed in Scott, 1991). Two holoenzyme subtypes called type I and type II are formed by the combination of RI or RII with the C subunits (Brostrom et al., 1971; Corbin et al., 1973).

Although many hormones use parallel pathways to activate PKA, some measure of specificity must be cryptically built into each signaling cascade to ensure that the correct pool of kinase becomes active in the right place and at the right time (Harper et al., 1985). Compartmentalization of the kinase has been proposed as a regulatory mechanism that may increase the selectivity and intensity of a cAMP-mediated hormonal response. To facilitate this process, up to 75% of type II PKA is targeted through association of the regulatory subunit (RII) with A-kinase anchoring proteins, called AKAPs (reviewed in Rubin, 1994 and Scott and McCartney, 1994). Additionally, there is now some indication that the type I PKA is also compartmentalized (Rubin et al., 1972; Skalhegg et al., 1994). In recent years, numerous AKAPs have been identified that target PKA to a variety of subcellular locations (Coghlan et al., 1993).

A-Kinase Anchoring Proteins (AKAPs)

Initially, anchoring proteins were identified as contaminating proteins that copurified with the RII after affinity chromatography on cAMP-sepharose (Sarkar et al., 1984). However, detailed study of AKAPs was made possible by the original observation of Lohmann et al. (1984) that many, if not all, of these associated proteins retain their ability to bind RII after they have been immobilized to nitrocellulose or similar solid-phase supports. As a result, the standard technique for detecting AKAPs is an overlay method which is essentially a modification of the Western blot (reviewed by Carr and Scott, 1992). Using the overlay technique we have surveyed various mouse, bovine, and human tissues and have detected AKAPs ranging in size from 21 to 300 kD (Carr et al., 1992*a*). From this type of study it would appear that a typical cell expresses 5–10 distinct AKAPs that presumably adapt the type II PKA for specific functions. Also, the expression of some AKAPs may be hormonally regulated, as treatment of granulosa cells with FSH induces the expression of an 80-kD anchoring protein (Carr et al., 1993). The RII overlay method has also been developed into an efficient interaction cloning strategy wherein cDNA expression libraries have been screened using recombinant RII as a probe. Collectively, these techniques have been used to probe RII/AKAP interactions and have allowed us to develop a model for the topology of the anchored PKA holoenzyme. This model, which is presented in Fig. 2, illustrates the essential features of AKAPs. Each anchoring protein contains two classes of binding site: a conserved "anchoring motif" which binds the R subunit of PKA, and a unique "targeting domain" which compartmentalizes the PKA-AKAP complex through association with structural proteins, membranes, or cellular organelles.

The Conserved RII Anchoring Motif

Since several anchoring proteins apparently bind to the same or overlapping sites on RIIα, it seemed likely that these molecules share a common RII-binding do-

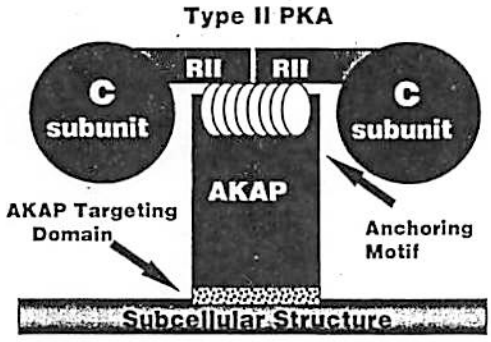

Figure 2. Topology of the anchored PKA holoenzyme complex; topology of anchored type II PKA. This model illustrates the essential features of AKAPs: (*A*) A conserved RII-binding site, and (*B*) a unique targeting domain for localization to intracellular sites.

main. Accordingly, one of the objectives of our expression cloning studies was to define a consensus RII-binding sequence that was common in all AKAPs. However, comparison of these sequences revealed no striking homology (Fig. 3 *A*) leading us to examine the RII-binding site in each anchoring protein for a conserved secondary structure binding motif. Computer aided secondary structure predictions of each putative RII-binding site showed a high probability for amphipathic helix formation (Fig. 3 *B*). The distinction between the hydrophobic and hydrophilic faces can be clearly seen when the sequences are drawn in a helical-wheel configuration (Carr et al., 1991). In each RII-anchoring protein there was a similar alignment of acidic residues throughout the hydrophilic face of each putative helix.

Analysis of Ht 31, a novel human thyroid RII anchoring protein of 1,035 amino acids that we cloned, identified a potential amphipathic helix between residues 494 to 509 (Carr et al., 1991). This sequence (Leu-Ile-Glu-Glu-Ala-Ala-Ser-Arg-Ile-

Figure 3. The conserved RII-binding motif on AKAPs. Association with the type II regulatory subunit of PKA occurs through a region of conserved secondary structure on the AKAPs. (*A*) Sequence alignment of the RII-binding regions on three AKAPs. Boxed areas represent regions of homology. (*B*) Each sequence is portrayed in a helical wheel projection (3.6 side-chains per turn) which is used to orient amino-acid side chains in an alpha helical conformation. Shaded areas represent the clustering on hydrophobic residues.

Val-Asp-Ala-Val-Ile-Glu-Gln) was 43% identical to a region within the RII-binding site of MAP 2. To determine whether residues 494-509 of Ht 31 were involved in RII binding, a 318 amino acid fragment representing residues 418 to 736 of Ht 31 was expressed in *E. coli*. Ht 31Δ 418-736 bound RIIα as assessed by solid-phase binding and gel-shift assays (Carr et al., 1991). To determine if an intact amphipathic helix was required for RII binding, a family of Ht 31 point mutants were produced in the Ht 31Δ 418-736 fragment. The introduction of proline into an α-helix conformation disrupts the secondary structure of the region and causes a 20° bend in the peptide backbone. The introduction of proline into the amphipathic helix region of Ht 31 diminished or abolished RIIα binding (Carr et al., 1991). Weak RIIα binding was observed with mutant Ht 31 Pro/Ala 498 which contained a mutation at position 5 in the putative helix region. No RIIα binding was detected with either Ht 31 Pro/Ile 502 or Ht 31 Pro/Ile 507. In contrast, proline substitution of Ala 522, which lies 12 residues downstream of the amphipathic helix region, had no apparent effect on RII binding. These results suggest that disruption of protein secondary structure between residues 498-507 of Ht 31 diminishes or abolishes RII binding. A peptide which spans the putative amphipathic helix region of Ht 31 binds RIIα with an affinity of approximately four, and circular dichromism analysis suggests it can adopt an α-helical conformation (Carr et al., 1992*a*).

PKA Anchoring In Vivo

So far, the majority of RII/AKAP interactions have been studied in vitro and under nonphysiological conditions. However, the high affinity of the amphipathic helix peptides for RII makes them ideal antagonists of PKA anchoring in vivo. The test system for these studies was the compartmentalization of PKA to the postsynaptic densities in hippocampal neurons where the kinase has easy access to the ionotrophic glutamate receptors. These receptors are central to the process of signal transduction across the synaptic membranes, and PKA-dependent phosphorylation is required to maintain the activity of AMPA/kainate responsive glutamate receptor channels (Greengard et al., 1991; Wang et al., 1991). The role of PKA in maintaining channel activity was confirmed by a gradual decline in whole-cell currents evoked by kainate (20 μM) recorded in the presence of ATP (20 μM) and 1 μM PKI (5-24) peptide, a potent and specific inhibitor of the catalytic subunit of PKA (61.8 ± 3.2% $n = 11$) (Fig. 4 *A*). To test the role of AKAPs in localizing the kinase near the channel, the anchoring inhibitor peptides (1 μM) were added to the whole cell pipette. The anchoring inhibitor peptide derived from two AKAPs, Ht 31 or AKAP79, inhibited AMPA/kainate currents to the same extent as the PKI peptide (64.9 ± 3.2% $n = 12$ and 68.8 ± 3.3% $n = 12$, respectively) (Fig. 4 *B*). The effects of PKI and the anchoring inhibitor peptides were not additive. However, the action of the Ht 31 peptide could be overcome by the C subunit of PKA (0.3 μM) suggesting that the anchoring inhibitor peptide interfered with PKA-dependent phosphorylation but did not directly inhibit the kinase (Fig. 4 *C*). In addition, the control peptide unable to block RII/AKAP interaction had no effect on kainate currents (85 ± 4.1% $n = 7$). Finally, currents evoked by AMPA (1 μM $n = 6$) behaved in the same manner as those evoked by application of kainate. Therefore, these results indicate that PKA localization is required for modulation of AMPA/kainate currents. These

studies represented the first physiological evidence of the importance of PKA anchoring in the modulation of a specific cAMP responsive event (Rosenmund et al., 1994). Recently, Catterall and colleagues (Johnson et al., 1994) have used these peptides to demonstrate that disruption of PKA anchoring close to the L-type Ca^{2+} channel is required to maintain the channel in the active state. The functional effect of phosphorylation of the receptors appears to be desensitization for their agonist.

The Delivery of Cell-soluble Bioactive Peptides

To perform the biochemical experiments it is often necessary to introduce bioactive peptides into cells. Two problems often mar this approach: the stability of the peptide and the solubility of the cell-soluble peptide form. A myristylated peptide based upon the Ht31 (493-515) anchoring inhibitor peptide was synthesized according to the methods of Eichholtz et al. (1993). These authors have previously shown that a myristylated pseudosubstrate peptide is cell soluble and inhibits PKC in vivo. The anchoring inhibitor peptide (10 mM) was dissolved in DMSO and diluted to a final concentration of 500 μM in 0.1% DMSO/PBS and incubated with living cultures of hippocampal neurons for 1 h at 37°C. Control experiments were performed with solutions of a nonmyristylated anchoring inhibitor peptide. Cultures were washed extensively in PBS and fixed with 3.7% formaldehyde for 5 min at room temp, washed in 100% acetone at −20°C for 1 min and incubated in a blocking solution of PBS containing 0.1% BSA for 30 min at room temp. Individual coverslips were stained for uptake of Ht31 (493-515) peptide with a peptide-specific antibody to the same sequence (1:200 dilution). Intracellular uptake of anchoring inhibitor peptide was detected by indirect immunofluorescence using a confocal microscope (Fig. 5). Confocal analysis of individual neurons was performed on nine focal planes (0.5 μm) to confirm that detection of immunofluorescence was predomi-

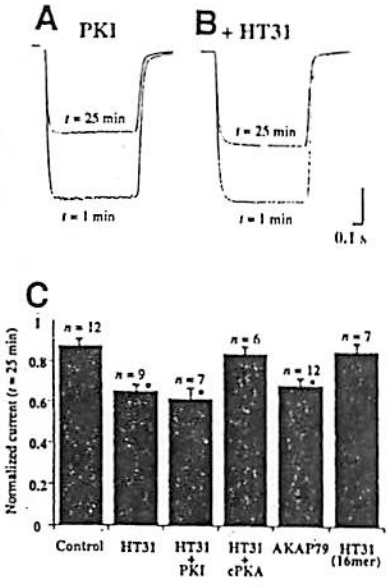

Figure 4. Displacement of PKA by anchoring inhibitor peptides blocks regulation of AMPA/kainate channels by PKA. (*A*) Inward currents evoked by kainate (20 μM) at 1 and 25 min after the start of whole cell recordings are superimposed. (*left*) The current decreased in the presence of the PKI(5-24) peptide (1 μM) which blocks kinase activity. (*B*) The current decreased to a similar extent in the presence of the Ht 31 peptide which is a competitive inhibitor of PKA anchoring. (*C*) Controls showing the amplitude of AMPA/kainate currents in the presence of ATP (5 mM) 25 min after application of various bioactive peptides and C subunit of PKA (0.3 μM). Peptide concentrations were Ht 3.1, (1 μM), PKI 5-24 (1 μM), and AKAP79 388-409 (1 μM). (Adapted from Rosenmund et al., 1994).

nantly intracellular. Increased amounts of the myristylated Ht31 (493-515) peptide were detected inside neurons (Fig. 5 *A*) when compared to cells incubated with the nonmyristylated form (Fig. 5 *B*). Control experiments incubated in the absence of peptide demonstrate the background level of staining (Fig. 5 *C*). This experiment suggests that myristylation is a viable means to introduce these peptides into living cells. Moreover, uptake of the myristylated peptide was primarily in the cell body and neurites which suggests that peptides will be available to compete with attachment to the PSD. It is possible that the myristal group targets these peptides to membranes which will be optimum for disruption of the AKAP79 signaling complex we believe is positioned close to the channels.

Other Enzyme Binding Sites on AKAPs: AKAP79/Calcineurin Interaction

In 1992, we cloned an anchoring protein called AKAP79 which is a component of the postsynaptic densities of neurons (Carr et al., 1992*b*). In accordance with the anchoring hypothesis, AKAP79 associates with the PSD to adapt the PKA for specific roles in postsynaptic events. Therefore, we proposed that AKAP79 must contain at least two functional domains (Scott and McCartney, 1994): a PKA binding region, and a targeting site responsible for association with proteins in the PSD. In an effort to identify AKAP-binding proteins, we used the yeast two-hybrid system (Fields and Song, 1989) to isolate cDNAs that encode proteins that associate with AKAP79 (Coghlan et al., 1995). While we expected to identify structural proteins, the two-hybrid method yielded a cDNA encoding another signaling enzyme, a cDNA for a murine β isoform of the calcineurin (CaN) A subunit. As anticipated, the two-hybrid system positively identified interactions between RII and itself (dimerization) and between RII and AKAP79 or Ht 31. These findings provide evidence for association of AKAP79 with CaN in yeast and suggest, because the AKAP also binds RII, the occurrence of a ternary complex between type II PKA, AKAP79, and CaN.

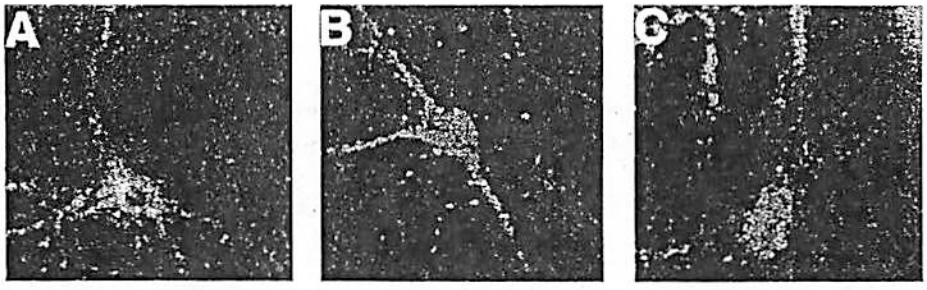

Figure 5. Uptake of myristylated peptide into cultured hippocampal neurons. Neurons were incubated with myristylated Ht31 (493-515) peptide. (*A*) Staining pattern for myr-Ht31 (493-515) peptide-treated neurons using affinity-purified peptide-specific antibodies (10 μg/ml). (*B*) Staining pattern for Ht31 (493-515) peptide-treated neurons using affinity-purified antibodies (10 μg/ml). (*C*) Staining pattern for neurons incubated with buffer alone, using affinity-purified antibodies (10 μg/ml).

Biochemical methods were employed to examine whether PKA and CaN were associated in mammalian brain. In initial experiments, calmodulin-binding proteins were isolated from bovine brain extracts by affinity chromatography and calcineurin was immunoprecipitated using affinity purified antisera against CaN A subunit. Immunoprecipitates were incubated with cAMP and the eluate was assayed for PKA activity by addition of ATP and Kemptide substrate. The specific activity of protein kinase was substantially increased by purification of a 30–60% ammonium sulfate fraction of the extract over calmodulin-agarose (28-fold; ±6, $n = 3$) followed by immunoprecipitation with CaN specific antibodies (123-fold; ±3.6). All of the protein kinase activity in the immunoprecipitate was cAMP-dependent and specifically inhibited by PKI peptide indicating that the catalytic (C) subunit of PKA was a component of the isolated complex. Coimmunoprecipitation of AKAP79 was confirmed by analysis of protein blots using a ^{32}P-RII overlay method. In complementary experiments, R subunits of PKA were isolated by affinity chromatography on cAMP-agarose. A proportion of the CaN present in the brain extract co-purified with R subunit and was eluted from the affinity matrix with 10 μM cAMP. Because recombinant CaN did not display any intrinsic cAMP binding activity, it was likely that the phosphatase was purified as part of a ternary complex with the AKAP and RII. Analysis of purified fraction by RII-overlay confirmed this hypothesis as AKAP79 was also present in the cAMP-agarose elution. Separate experiments were performed using a PKA anchoring inhibitor peptide, specifically displaced AKAPs from RII bound on the affinity column (Fig. 6). The peptide eluted both CaN (Fig. 6 *A*) and AKAP79 (Fig. 6 *B*), while R subunit remained on the affinity column and was subsequently eluted with cAMP (Fig. 6 *C*). Combined, these results confirm the simultaneous association of CaN and type II PKA with the AKAP.

Our model for colocalization of PKA and CaN through AKAP79 implies that the AKAP contains distinct sites for kinase and phosphatase binding. Residues 88 to 102 of AKAP79 were considered likely to comprise the CaN binding site due to homology with a region of the immunophilin FKBP-12 that contains determinants for CaN association. Binding of FKBP-12 to CaN is dependent on the immunosuppressant drug FK-506 and results in inhibition of the phosphatase. Considering our finding that CaN was inactive when isolated as a complex with the AKAP, we ex-

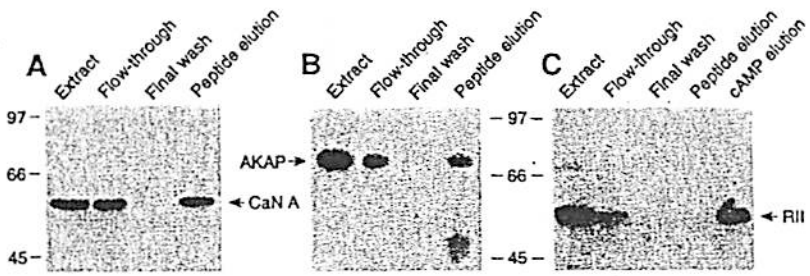

Figure 6. Copurification of calcineurin and PKA from brain. R subunits were purified from crude extracts of bovine brain by affinity chromatography with cAMP-agarose. The AKAP/CaN complex was eluted with Ht31 (493-515) peptide. (*A*) Protein immunoblots were probed with affinity purified antibodies against calcineurin. (*B*) ^{32}P RII; (*C*) affinity-purified RII antibodies.

amined the effects of recombinant AKAP79 on CaN phosphatase activity. The AKAP inhibited both full-length CaN (Ca^{2+}/calmodulin dependent) and a truncated, constitutively active form of CaN, called CaN_{420} (Ca^{2+}/calmodulin independent), in a noncompetitive manner ($K_i = 4.2 \pm 1.8$ μM; $n = 3$) with respect to phosphorylated RII-substrate peptide. Furthermore, a synthetic peptide, corresponding to AKAP79 residues 81-102, inhibited both CaN forms, whereas the Ht31 (493-515) peptide was not an inhibitor of CaN. The observed inhibition was specific for calcineurin; AKAP79 (81-102) peptide did not significantly affect the activity of protein phosphatases 1 or 2A at peptide concentrations as high as 0.4 mM. Although CaN-binding sites on AKAP79 and FKBP-12 are similar, their differences may have functional significance: FK-506 (2 μM) did not affect the potency of inhibition, and recombinant AKAP79 did not display peptidyl prolyl isomerase activity toward a fluorescent peptide substrate. Collectively, these findings suggest that CaN is localized by AKAP79 in its inactive state in a manner analogous to AKAP-bound PKA. While the mechanism for activation of PKA by cAMP is apparent, regulation of AKAP-CaN association is likely to involve multiple factors.

AKAP79/Protein Kinase C Interaction

In neurons, PKA and CaN are both localized to postsynaptic densities (PSD) by association with AKAP79 which positions both enzymes close to key neuronal substrates. Because other neuronal signaling enzymes are present at the PSD (Wolf et al., 1986), we investigated their potential to associate with the anchoring protein AKAP79. Protein kinase C (PKC), a family of ser-thr kinases, is tethered to the PSD through association with binding proteins (Wolf et al., 1986). We used a solid phase binding assay (overlays) with PKC as a probe on bovine brain extracts to detect several PKC-binding proteins, including a protein that migrated with the same mobility as a prominent RII-binding protein of 75 kD. This band corresponds to AKAP75, the bovine homolog of AKAP79. This result indicated that the anchoring protein could bind both RII and PKC. Indeed, recombinant AKAP79 bound to PKC in the presence of Ca^{2+} and phosphatidylserine. This suggests that the AKAP binds in a different manner to other PKC-substrate/binding proteins such as MARCKS (myristylated alanine-rich C kinase substrate) and g-adducin, for which phosphorylation regulates association with PKC (Hyatt et al., 1994). We mapped the PKC-binding region by screening a family of AKAP79 fragments. Fragments encompassing the first 75 residues of AKAP79 bound PKC, whereas COOH-terminal fragments containing the RII and CaN-binding regions did not, thus implying that PKC binds to AKAP79 at a site that is distinct from those bound by RII and CaN.

Because basic and hydrophobic regions are determinants for binding of certain proteins to PKC (Liao et al., 1994) we focused our attention on a region located between residues 31 to 52 of AKAP79 (Fig. 7 *A*). A peptide encompassing this region specifically blocked the interaction of AKAP79 with PKC in the overlay assay but did not affect RII-binding to the AKAP (Fig. 7 *B*). Conversely, the RII anchoring inhibitor peptide (AKAP79 388-409) did not affect PKC-binding but did block interaction with RII (Fig. 7 *C*). This indicates that residues 31 to 52 represent the principal determinants for PKC-binding. Many kinases or phosphatases bound to anchoring proteins are maintained in an inactive state (Faux and Scott, 1996*b*). Ac-

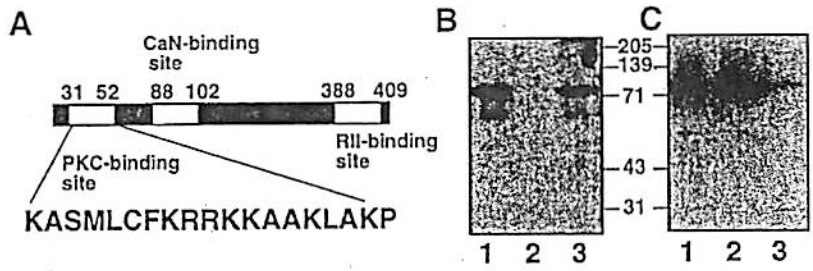

Figure 7. Inhibition of PKC activity by AKAP79. (*A*) Schematic representation of AKAP79 showing putative binding sites for PKC, CaN, and RII. The amino acid sequence for residues 31 to 52 is indicated. (*B*) Recombinant AKAP79 was blotted and PKC overlays were done in the absence (lane *1*) and presence of either 1.5 μM AKAP79 (31-52) (lane *2*) or 1.5 μM RII-anchoring inhibitor peptide AKAP79 (390-412) (lane *3*) with ~12.5 nM PKC. (*C*) ^{32}P-RII (100 cpm/μl) overlays were done under the same conditions as in *B*.

cordingly, recombinant AKAP79 protein inhibited PKC activity with a half-maximal inhibition (IC_{50}) value of 0.35 ± 0.06 μM (n = 3). In addition, the AKAP79 peptide (residues 31 to 52) and a recombinant AKAP79 fragment (residues 1 to 75) inhibited PKC activity with IC_{50} values of 2.0 ± 0.6 μM (n = 4) and 1.6 ± 0.3 μM (n = 4), respectively. In contrast, the 31-52 peptide did not inhibit the activity of the catalytic subunit of PKA. Inhibition of PKC activity by the 31-52 peptide was mixed with an apparent inhibition constant (K_i) of 1.41 ± 0.28 μM (n = 3). The secondary plot of

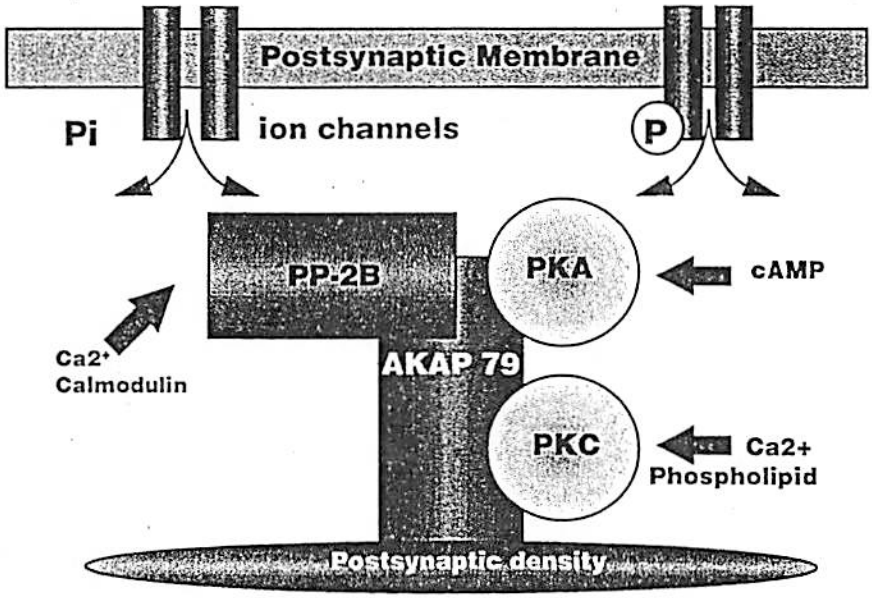

Figure 8. The AKAP79 signaling complex. The anchoring protein AKAP79 is attached to the postsynaptic densities and maintains PKA, PKC, and calcineurin close to substrates in the postsynaptic membrane.

the Michaelis constant divided by the maximal velocity (K_m/V_{max}) as a function of inhibitor concentration was nonlinear, suggesting binding at more than one site. Therefore, we propose that PKC is localized and inhibited by AKAP79 in a Ca^{2+}- and phosphatidylserine-dependent manner, and peptide studies suggest that residues 31-52 represent a principal site of contact.

Conclusions

Anchoring proteins have emerged as a new class of regulatory molecule which contribute to the organization and specificity of signal transduction pathways. The broad specificity kinases and phosphatases seem to use these proteins not only to direct their catalytic subunits to particular parts of the cell but also to enhance specificity towards preferred substrates. This may represent a mechanism to prevent indiscriminate phosphorylation or dephosphorylation of phosphoproteins by these broad specificity enzymes. A variation on this theme seems to be multifunctional anchoring proteins such as AKAP79, which package several signaling enzymes into multiprotein complexes. This may prove to be an efficient means for controlling the phosphorylation state of a given protein in response to multiple intracellular signals such as Ca^{2+}, phospholipid, and cAMP. Moreover, the structure of AKAP79 is modular, in that deletion analysis, peptide studies and coprecipitation techniques have demonstrated that each enzyme binds to a distinct region of the anchoring protein. Targeting of the AKAP79 signaling complex to the postsynaptic densities suggests a model for reversible phosphorylation in which the opposing effects of kinase and phosphatase action are colocalized in a signal transduction complex by association with a common anchor protein (Fig. 8). Potential substrates for the AKAP79 transduction complex are likely to be synaptic receptor/channels and may include AMPA/kainate receptors and Ca^{2+} channels which have recently been shown to be modulated by AKAP-targeted PKA, and NMDA receptors which are activated by PKC and attenuated by calcineurin (Klauck and Scott, 1995).

Acknowledgments

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