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Coordination of cAMP Signaling Events through PKA Anchoring

I. Introduction

The efficacy of signal transduction events, such as those mediated by the second messenger cyclic 3′,5′-adenosine monophosphate (cAMP), is often taken for granted. Yet the mechanism with which extracellular effectors such as hormones, prostaglandins, or neurotransmitters induce the movement of signals from the inner face of the plasma membrane to specific intracellular targets still remains somewhat enigmatic, particularly when one considers the large number of polypeptides devoted to this process within a eukaryotic cell (Hunter, 1995). This dilemma is compounded by the finding that the cAMP-dependent protein kinase holoenzyme, the principal receptor for cAMP, is able to phosphorylate a wide array of cellular substrates. Thus, mechanisms must clearly exist to organize the correct repertoire of signaling molecules into coordinated units that allow only a subset of the PKA substrates to become phosphorylated (Pawson and Scott, 1997).

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One mechanism involves the restriction of PKA to localized sites of action where it can only phosphorylate those substrates in its immediate vicinity. This is achieved in one of two ways: compartmentalized accumulation of the second messenger cAMP, or anchoring of the kinase to subcellular structures and organelles (Faux and Scott, 1996a; Pawson and Scott, 1997). In this chapter we review accumulating data suggesting that the subcellular location of the cAMP-dependent protein kinase is regulated in part through association with a family of A-kinase anchoring proteins called AKAPs.

II. The cAMP-Dependent Protein Kinase

When cAMP was first discovered as a soluble second messenger of hormone stimulated events in the late 1950s (Sutherland and Rall, 1957), it was soon recognized that its primary action in eukaryotic cells was to activate a cAMP-dependent protein kinase (PKA) (Taylor *et al.*, 1990). The PKA holoenzyme is a heterotetramer composed of a regulatory (R) subunit dimer that maintains two catalytic (C) subunits in a dormant state (Corbin and Keely, 1977; Corbin *et al.*, 1973; Potter *et al.*, 1978; Potter and Taylor, 1979). Holoenzyme dissociation ensues upon binding of cAMP to tandem sites in each R subunit (Su *et al.*, 1993, 1995). This alleviates an autoinhibitory contact that releases the active C subunit (Gibbs *et al.*, 1992; Wang *et al.*, 1991). The active kinase is then free to phosphorylate substrates on serine or threonine residues which are presented in a sequence context of Arg-Arg-Xaa-Ser/Thr or Lys-Arg-Xaa-Xaa-Ser/Thr (Kemp *et al.*, 1977; Kemp and Pearson, 1990). Given the frequent occurrence of these sequence motifs in many proteins, it was soon reasoned that unrestricted access of the C subunit to its substrates would lead to indiscriminate phosphorylation. Consequently, several regulatory mechanisms are in place to ensure that cAMP levels and kinase activity are tightly controlled (Adams *et al.*, 1991, 1992; Bacskai *et al.*, 1993; Barsony and Marks, 1990). Second messenger levels are controlled by a balance of adenylyl cyclase (AC) and phosphodiesterase (PDE) activities that generate gradients of cAMP emanating from the plasma membrane (Beavo *et al.*, 1994; Tang and Gilman, 1992). Supplementary signal terminating mechanisms, such as desensitization of AC or compartmentalized activation of PDEs, ensure localized reduction of the second messenger (Cooper *et al.*, 1995; Mons *et al.*, 1995; Shakur *et al.*, 1993; Smith *et al.*, 1996). Although access to cAMP is the primary requirement for PKA activation, additional factors are responsible for driving holoenzyme reformation and returning the kinase to the inactive state. The R subunits are expressed in excess over C subunits favoring rapid reformation of the holoenzyme when cAMP levels return to the basal state (Amieux *et al.*, 1997). In addition, the ubiquitous heat stable inhibitor, PKI, may well serve as a fail-safe device that mops up free C subunit (Krebs and Beavo, 1979).

PKI also facilitates export of the C subunit from the nucleus, which is devoid of R subunits; this suggests a more specific function as a signal terminator for nuclear phosphorylation events (Fantozzi *et al.*, 1992; Wen *et al.*, 1994, 1995).

For some time now it has been thought that the cellular specificity of PKA signaling is related to the existence of multiple C and R subunit isoforms. In mammals, three C subunit isoforms (α, β, and γ) exist, and, although there are subtle differences in the kinetic profiles and cAMP sensitivities for Cα and Cβ containing holoenzymes, these two predominant C subunit isoforms are virtually indistinguishable with respect to substrate specificity and interaction with R subunits (Gamm *et al.*, 1996; Scott, 1991; Taylor *et al.*, 1990). In contrast, the dimeric R subunits exhibit both distinct cAMP binding affinities and differential localization within cells (Corbin *et al.*, 1973, 1975). The type I PKA holoenzyme, which contains either RIα or RIβ, is predominantly cytoplasmic and more sensitive to cAMP than type II PKA. In contrast, up to 75% of the type II PKA is targeted to certain intracellular sites through association of the RII subunits, RIIα or RIIβ, with cellular binding proteins now known as anchoring proteins (for review, see Rubin, 1994; Dell'Acqua and Scott, 1997). Thus, it has been proposed that differences in subcellular targeting of type I and type II PKA are additional factors contributing to specificity in cellular responses.

III. AKAPs

The first RII-binding proteins were identified in the early 1980's by Vallee, De Cammili, Rubin, and Erlichman as contaminating proteins that which copurified with RII after affinity chromatography on cAMP-sepharose (reviewed by Dell'Acqua and Scott, 1997). However, detailed study of these proteins was made possible by the observation of Lohmann and colleagues that many, if not all, of these associated proteins retain their ability to bind RII after they have been immobilized on nitrocellulose filters (Lohmann *et al.*, 1984). As a result, the standard technique for detecting RII-binding proteins is an overlay method that is a slight modification of the Western blot but using radiolabeled RII subunit as a probe (reviewed by Hausken *et al.*, 1997). Using this technique, RII-binding bands ranging in size from 15 to 300 kDa have been detected in a variety of tissues, and it would appear that a typical cell expresses 5–10 distinct binding proteins (Carr *et al.*, 1992a). The RII overlay method has also been refined into an efficient interaction cloning strategy wherein cDNA expression libraries are screened using RII as a probe. This has led to the cloning of numerous RII-binding proteins (Bregman *et al.*, 1989; Carr *et al.*, 1992b; Carr and Scott, 1992; Coghlan *et al.*, 1994; Dransfield *et al.*, 1997b; Lester, 1996; McCartney *et al.*, 1995; Nauert *et al.*, 1997; Lin *et al.*, 1995; Fraser *et al.*, 1998). More

recently, these RII-binding proteins were renamed A-kinase anchoring proteins, or AKAPs, to account for their proposed PKA targeting function (Hirsch *et al.*, 1992). A model is presented in Fig. 1 that illustrates the essential domains of AKAPs. Each anchoring protein contains two types of binding site: a common "anchoring motif" that binds the R subunit dimer of PKA, and a unique "targeting domain" that directs the subcellular localization of the PKA–AKAP complex through association with structural proteins, membranes, or cellular organelles.

It was formerly believed that AKAPs exclusively target the type II PKA holoenzyme in which the C subunit is bound to the RII isoform of the regulatory subunit. However, two dual function AKAPs have now been discovered that bind RI or RII (Huang *et al.*, 1997a, 1997b). Although *in vitro* studies indicate that RI binds several AKAPs with a 100-fold lower binding affinity than RII, the submicromolar binding constant interaction lies within the physiological concentration range of RI and AKAPs inside cells. Thus, type I PKA anchoring may be relevant under certain conditions where RII concentrations are limiting, such as in experiments on RIIα knockout mice where Ca^{2+} channel modulation is more sensitive to anchoring inhibitor reagents than in wild-type animals (Burton *et al.*, 1997). Another recently recognized property of AKAPs is that some of the anchoring proteins simultaneously bind PKA and one or more other signaling enzymes. These multivalent AKAPs serve as scaffolds for the assembly of multienzyme signaling complexes consisting of several kinases and phosphatases (Faux and Scott, 1996b). Despite some variations between individual AKAPs, all of these proteins represent a functionally related family of proteins that at least bind PKA and target their complement of kinases and phosphatases to specific subcellular structures. Consequently, the remainder of this chapter will focus on two areas of AKAP research: (1) the role of the anchoring motif as a tool to disrupt PKA anchoring inside cells; and (2) the mapping of AKAP targeting signals that have been used to functionally redirect PKA to specific intracellular locations.

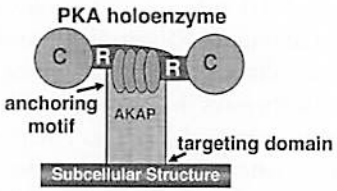

FIGURE 1 Model of the anchored PKA holoenzyme complex. Binding surfaces on the AKAP for association with PKA (anchoring) and for interaction with subcellular organelles or structures (targeting) are indicated.

A. The RII Anchoring Motif

Early work in the field focused on mapping the sites on RII required for interaction with AKAPs. Initially, the minimum region of RII required to bind MAP2 and AKAP75 was defined by screening a family of deletion mutants and chimeric proteins by the overlay assay (Scott *et al.*, 1990; Luo *et al.*, 1990). These studies concluded that RII dimerization was a prerequisite for anchoring and that AKAP-binding required the first 45 residues of RII (Hausken *et al.*, 1994; Luo *et al.*, 1990; Scott *et al.*, 1990). Subsequently, the localization and dimerization determinants were mapped to distinct subsites within this region (Hausken *et al.*, 1994, 1996). Deletion of residues 1 to 5 abolished the anchoring function but had no qualitative effect upon dimerization, and site-directed mutagenesis studies led to the identification of isoleucines at positions 3 and 5 as essential determinants for association with AKAPs (Hausken *et al.*, 1994, 1996). Since leucines and isoleucines are also crucial determinants of the reciprocal binding surface on the AKAP (Glantz *et al.*, 1993) it is possible that RII/AKAP docking may be analogous to the hydrophobic interactions that maintain a leucine zipper in transcription factors such as CEBP and CREB. However, it must be noted that the protein–protein interactions required for RII/AKAP interaction are more elaborate and involve three polypeptide chains, i.e., two RII protomers and a binding surface on the AKAP (Fig. 1). This view is supported by the stoichiometry of the interaction, which suggests that one AKAP binds per RII dimer (Carr *et al.*, 1992a). Furthermore, additional AKAP-binding determinants have been mapped between residues 11 to 25 of each RII molecule (Li and Rubin, 1995).

The rudimentary analysis conducted on RII/AKAP interaction has more recently been confirmed and extended by structural studies that have solved the structure of the AKAP-binding and dimerization surfaces on RIIα. The solution structure of an amino terminal fragment encompassing the first 45 residues of RIIα has been solved by nuclear magnetic resonance (NMR) spectroscopy. Both RII protomers form an antiparallel four-helix bundle with the principal AKAP-binding determinants located within an α-helix between residues 8 and 14, while a second helix between residues 28 and 42 maintains the dimerization contact. In this topology, isoleucines 3 and 5 form hydrophobic regions at either end of the AKAP-binding pocket that would permit their interaction with a reciprocal binding surface on the AKAP (Newlon *et al.*, 1997). Although these residues are principal anchoring determinants, further analysis of an RII/AKAP peptide complex suggests that numerous side chains within the AKAP-binding surface contact the anchoring protein. This may explain the nanomolar binding affinity of RII/AKAP interactions that have been measured by a variety of analytical techniques (Carr *et al.*, 1991, 1992a, 1992b; Hausken *et al.*, 1997). Furthermore,

modeling studies suggest that a helical region common to all AKAPs fits snugly into the AKAP-binding surface.

Another level of specificity in PKA signaling may be achieved through the differential localization of RIIα or RIIβ by association with isoform-selective AKAPs. Alternatively, different AKAPs may be selectively expressed in cell types where one PKA isoform predominates. It was reported that RIIα had a sixfold preference for MAP2, whereas RIIβ had a twofold preference for AKAP75 (Leiser *et al.*, 1986). It has also been shown that follicle-stimulating hormone (FSH) treatment of rat granulosa cells induces an 80-kDa RIIα-selective AKAP (Carr *et al.*, 1993). A structural explanation for this differential binding affinity can be reasoned from analysis of the RII 1-45 structure. Sequences of the first 10 amino acids of RIIα and RIIβ are almost identical except for a pair of prolines at positions 6 and 7 in RIIα that is not present in RIIβ. A proline pair at positions 6 and 7 increases the rigidity of the helix–turn–helix motif that forms the AKAP-binding pocket (Newlon *et al.*, 1997). In fact, proline 6 may increase RIIα affinity for certain AKAPs through direct contact with the anchoring proteins. It is interesting to note that another proline pair exists in RIIα at positions 24 and 25 that proceeds the second helix. Interestingly, proline 26 is not conserved in RIIβ and is replaced with an alanine residue. This change is likely to increase the flexibility of the second helix and consequently may alter the dimerization interface in RIIβ. Structure/function studies are currently under way to assess the contribution of this region in the AKAP-binding affinity of RIIβ.

B. The PKA Anchoring Site on AKAPs

Reciprocal studies have identified a common site on AKAPs that binds the R subunit. Deletion analyses located the RII-binding sequences of MAP2 and AKAP150 to short regions of continuous amino acid sequence (Obar *et al.*, 1989; Rubino *et al.*, 1989). However, the nature of the RII-binding motif remained unclear until a human thyroid anchoring protein, called Ht31, was identified (Carr *et al.*, 1991, 1992a). The RII-binding sequence of Ht31 exhibited sequence similarities to both MAP2 and AKAP150 and was predicted to form an α-helix. Helical wheel projections of all three sequences exhibited a striking segregation of hydrophobic and hydrophilic side chains. This led to the proposal that the RII-binding motif of Ht31 and other AKAPs involves an amphipathic helix (Carr *et al.*, 1991). Subsequent studies performed on Ht31 and AKAP79, the human homologue of AKAP150, demonstrated a requirement for this region in RII-binding (Carr *et al.*, 1991, 1992b). The role of helical secondary structure in RII–AKAP interactions was supported by demonstrating that substitution of proline, a residue that perturbs helix formation, at various positions within the RII binding domain abolished RII-binding (Carr *et al.*, 1991, 1992a). These findings were consolidated by the synthesis of peptides encompassing the

predicted helical region of Ht31 that were shown to bind either RII or the type II PKA holoenzyme with nanomolar affinity (Carr *et al.*, 1992a; Hausken *et al.*, 1997). Subsequent studies have independently confirmed that similar regions on several other AKAPs are essential determinants for RII-binding (Coghlan *et al.*, 1994; Dransfield *et al.*, 1997b; McCartney *et al.*, 1995; Nauert *et al.*, 1997). The high affinity of these interactions has important consequences for the intracellular localization of PKA. First, the K_D for the RII/AKAP interaction has been calculated from 1 to 11 nM by a variety of analytical methods (Hausken *et al.*, 1997). This affinity constant is well within the intracellular concentration ranges of RII and most AKAPs, suggesting that the RII/AKAP complex will be favored *in situ*. Secondly, the PKA holoenzyme binds Ht31 with the same high affinity as the RII dimer. Thus, the PKA holoenzyme will be anchored in cells when cAMP is at basal levels. Although the involvement of an amphipathic helix has not been definitively proven, analysis of the RII 1-45 structure suggests that an α-helix is the optimal structure to fit into the AKAP-binding pocket (M. Newlon and P. Jennings, personal communication).

C. The Use of AKAP-Derived Peptides inside Cells

Knowledge of the RII-binding domains on several AKAPs has allowed the generation of reagents that alter PKA anchoring within cells. Peptides encompassing the amphipathic helix region of Ht31 (residues 493–515) effectively compete for RII-AKAP interaction *in vitro* and disrupt the subcellular localization of PKA inside cells (Rosenmund *et al.*, 1994). Perfusion of cultured hippocampal neurons with these "anchoring inhibitor peptides" caused a time-dependent decrease in AMPA/kainate-responsive currents, whereas perfusion of control peptides, which were unable to compete for RII-binding, had no effect on channel activity (Rosenmund *et al.*, 1994). Additional controls confirmed that the effects emanated from PKA as perfusion of PKI peptides, which block kinase activity, caused a decrease in channel activity, whereas microinjection of excess C subunit overcame the anchoring inhibitor effect. Collectively, these findings suggested that the Ht31 peptide displaced PKA from anchored sites close to the AMPA/kainate channels, thereby decreasing the probability of channel phosphorylation. Parallel studies by Catterall and colleagues have subsequently shown that Ht31 peptide-mediated disruption of PKA anchoring modulates L-type Ca^{2+} channels in skeletal muscle (Johnson *et al.*, 1994, 1997). Likewise, peptide-mediated disruption of PKA targeting has been implicated in the regulation of smooth muscle calcium activated potassium (K_{CA}) channels (Wang and Kotlikoff, 1996). K_{CA} channels were recorded from tracheal myocytes, showing that introduction of the anchoring inhibitor peptide Ht31 blocked stimulation of the channels that were induced by ATP, whereas the Ht31 control peptide had no adverse effect in channel stimulation. Taken together, each of

these studies provide convincing evidence that PKA anchoring may facilitate preferential modulation of physiological PKA substrates. However, there are technical limitations associated with the introduction of bioactive peptides into cells. Although microinjection or microdialysis is suitable for peptide delivery into single cells, the uptake of cell-soluble peptide analogues is necessary to affect many cells. Accordingly, cell-permeant anchoring-inhibitor peptides and Ht31 expression plasmid have been developed to efficiently displace the intracellular location of PKA in a variety of cell types. As will be discussed in detail later, we have used cell soluble versions of the Ht31 peptides to define a role for PKA anchoring in hormone-mediated insulin release from pancreatic beta cells.

D. The Role of PKA Anchoring in GLP-1 Mediated Insulin Secretion

Insulin secretion requires the coordinated action of metabolites, hormones, and neurotransmitters and is regulated by a variety of second-messenger-mediated signaling events that alter the dynamic balance in kinase and phosphatase activity in pancreatic beta cells (Ammala *et al.*, 1994; Sjoholm, 1995). For example, a recently identified hormone, glucagon-like peptide 1 (GLP-1), potentiates glucose-mediated insulin secretion through activation of PKA to favor exocytosis of insulin secretory granules (Drucker *et al.*, 1987; Thorens, 1992; Yaekura *et al.*, 1996). In one study we demonstrate that the subcellular targeting of PKA by a family of targeting proteins called AKAPs is an additional mechanism in the regulation of hormone-mediated insulin secretion (Lester *et al.*, 1997). Using cell-soluble Ht31 anchoring inhibitor peptides and expression of Ht31 plasmid, we demonstrated that the correct targeting of PKA is a determinant in the cAMP response to GLP-1 that induces insulin secretion from pancreatic islets and related cell lines. Lipofectamine was used as a delivery reagent to introduce Ht31, Ht31P control, or a PKA inhibitor peptide, PKI 5-24, into primary rat islets. Concomitant changes in glucose mediated insulin secretion and GLP-1 mediated insulin secretion were monitored by radioimmunoassay. The response of islets treated with any of the bioactive peptides to glucose was significantly different (Fig. 2A). Nontreated islets had similar glucose-mediated insulin secretion of 4.5 ng/well ± 0.57 (Fig. 2A), whereas islets treated with insulinotropic hormone GLP-1 that activates PKA exhibited a (3.5 ± 0.5) fold further increased insulin secretion (Fig. 2B) (Cullinan *et al.*, 1994; Gromada *et al.*, 1995a, 1995b; Yada *et al.*, 1993). Application of the PKA inhibitor peptide (PKI 5-24) blocked the GLP-1 effect (0.7 ± 0.15), confirming a role for the kinase in this process (Fig. 2B). Interestingly, the GLP-1 effect was blocked (0.9 ± 0.1 fold) in islets treated with the Ht31 anchoring inhibitor peptide, whereas the control peptide (Ht31P) had no effect (2.8 ± 0.3 fold increase; Fig. 2B). Similar results were obtained when

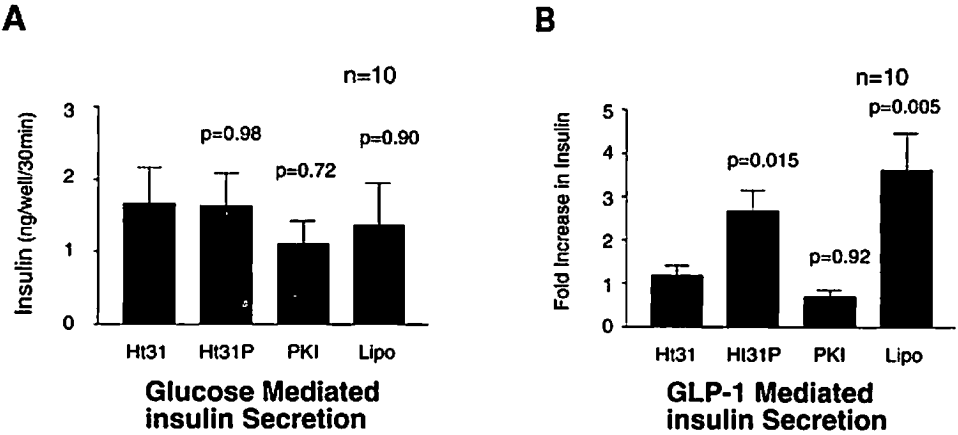

FIGURE 2 Disrupting PKA anchoring inhibits GLP-1 mediated insulin secretion in primary islets. Peptides were introduced into primary cultures of pancreatic islets to assess the role of PKA anchoring on insulin secretion. (A) Glucose-mediated insulin secretion in islets treated with peptides or a lipofectamine control (indicated below each bar) was measured in the culture media by radioimmunoassay. Data from 10 experiments are shown. (B) Changes in GLP-1 mediated insulin secretion were measured by similar methods and are presented as the fold increase over glucose mediated insulin secretion. Data from 10 experiments are indicated.

cell-soluble myristoylated derivatives of these peptides were used in the absence of lipofectamine. Additional controls showed that none of the peptides affected cAMP production in response to GLP-1 or inhibited PKA C subunit activity toward the heptapeptide substrate Kemptide. In sum, these results suggest that disruption of PKA-AKAP targeting attenuates GLP-1 stimulated insulin secretion in pancreatic beta islets.

In order to assess the role of PKA anchoring in regulating insulin secretion by an alternate method, a clonal rat beta cell line, RINm5F, was transfected with plasmids encoding a soluble Ht31 fragment (residues 418–718) or with a mutant form, Ht31P, which was unable to bind RII. Immunochemical analysis demonstrated the diffuse expression of the Ht31 and Ht31P proteins in the appropriate cells while only background staining was seen in the wild-type RINm5F cells (Fig. 3, top panels). The intracellular location of RII was concentrated at perinuclear regions in cells expressing the Ht31P or RINm5F cells (Fig. 3, center column). In contrast, RII was more evenly distributed throughout the cytoplasm in cells expressing the Ht31 fragment (Fig. 3, top-center panel). This redistribution of RII by expression of the anchoring inhibitor protein confirms the *in vitro* disruption of RII–AKAP interactions in islets and RINm5F cells and is consistent with the role of these compounds to alter the subcellular location of the type II PKA holoenzyme (Carr *et al.*, 1991, 1992a). Since RINm5F cells have reduced sensitivities to GLP-1 and glucose (Watanabe *et al.*, 1994), we used dibutyryl cAMP

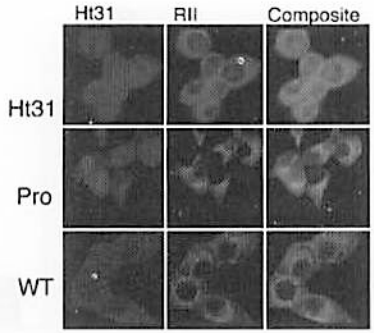

FIGURE 3 Recombinant anchoring inhibitor proteins displace PKA inside cells. The subcellular distribution of Ht31 and RII in RINm5F cells was detected immunochemically using a Leitz Fluovert confocal photomicroscope. The cell lines are indicated next to each row. Ht31 staining (left column), RII staining (center column), and composite images of both signals (right column) are presented.

as an index of GLP-1 action and membrane depolarization with potassium chloride (KCl) as an indicator of PKA independent insulin secretion. The response to 40 mM KCl was similar in all three groups (Fig. 4A), whereas cAMP-mediated insulin secretion was suppressed (1.2 ± 0.03)-fold in cells expressing the active Ht31 fragment (Fig. 4B). However, control cells expressing Ht31P or transfected with vector alone had normal cAMP-mediated insulin responses (Fig. 4B) of approximately (3.0 ± 0.8)-fold above basal.

In an effort to identify how PKA anchoring facilitates cAMP-mediated insulin secretion, we monitored the intracellular calcium response of RINm5F cells expressing Ht31, Ht31P, or vector alone. Increased intracellu-

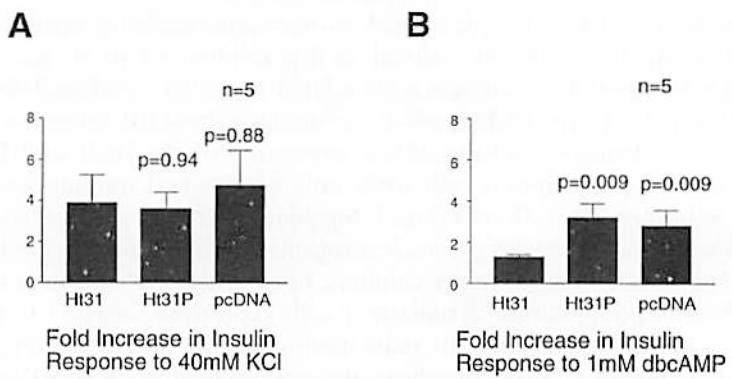

FIGURE 4 Anchoring inhibitor expression blocks cAMP-mediated insulin secretion in beta cells. (A) KCl-mediated insulin secretion and (B) cAMP-mediated insulin secretion by radioimmunoassay. Agonist-induced changes in insulin secretion are indicated as the fold increase over basal levels. Sample sources are indicated below each bar and P values for the mean ± SEM ($n = 5$).

lar calcium levels were detected in response to depolarizing amounts of KCl in all cells (Figs. 5A and 5B). However, the Ht31 expressing cells consistently failed to show an increase in intracellular calcium upon application of dibutyryl cAMP (Figure 5C&D). This suggests that anchored pools of PKA may facilitate cAMP-mediated phosphorylation events that modulate calcium fluxes from intracellular or extracellular sources. Data in pancreatic beta cells have suggested that the L-type Ca^{2+} channel is a site for PKA phosphorylation. This is supported by evidence that PKA anchoring augments Ca^{2+} channel modulation and by data showing that the beta cell L-type Ca^{2+} channel is phosphorylated by the kinase in RINm5F cells (Gray *et al.*, 1997; Johnson *et al.*, 1994, 1997). As will be discussed later in this

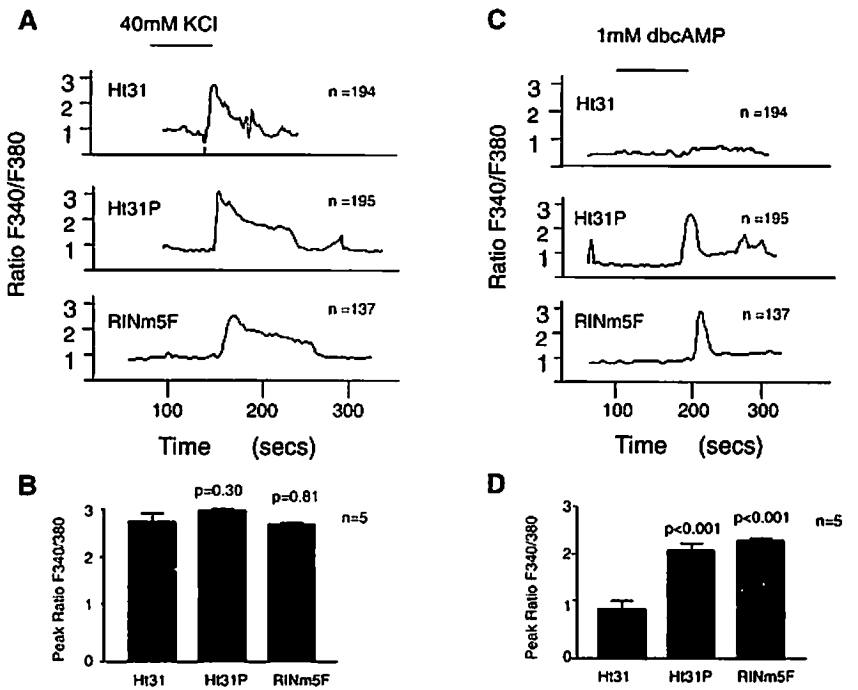

FIGURE 5 Disrupting PKA anchoring blocks rise in intracellular calcium in RINm5F cells. The effect of PKA anchoring on changes in intracellular calcium levels in RINm5F cells and cell lines expressing AKAP fragments was assessed by single-cell microfluorimetry on a Zeiss Axiophot scope. Changes in intracellular calcium (340/380 ratio) were detected by dual wavelength excitation at 340/380 nm with emission at 510 nm in response to (A) 40 mM KCl and (C) 1mM db-cAMP. Recordings were obtained every 4 s in a minimum of 15 cells per field. A representative response is shown for each experimental group and the total number of cells evaluated is indicated in each panel. The cell source is indicated for each sample group. The time of agonist infusion is indicated by the solid bar. The peak F340/380 ratio (from 5 individual experiments) is graphically depicted for the response to (B) 40 mM KCl and (D) 1 mM dbcAMP. Two-tailed *P* values comparing the mean of Ht31 to the other cell lines are indicated above the appropriate bars.

chapter, one site of PKA anchoring may be at or proximal to the L-type calcium channel.

Overall, the conclusions from this study demonstrate the utility of the anchoring inhibitor reagents as cell-based tools to disrupt the intracellular location of PKA by competing for R subunit AKAP interaction. However, despite demonstrating the usefulness of these peptides and providing compelling evidence that the proline derivatives of these compounds are ineffective in the disruption of PKA anchoring, we cannot rule out the possibility that the active anchoring inhibitor reagents are producing some secondary effects *in vivo*. Furthermore, these reagents only serve as vectors to globally disrupt PKA anchoring inside cells. As will become apparent in the latter sections of this chapter, specific subcellular targeting of PKA is achieved through understanding the unique targeting domains on the anchoring proteins and the heterologous expression of compartment-specific AKAPs.

E. AKAP Targeting Interactions

The PKA-anchoring model presented in Fig. 1 proposes that AKAPs should contain a unique targeting site that directs the association of PKA or the signaling complex to subcellular structures. In essence, the targeting domain is a fundamental feature of each AKAP, as it confers specificity by tethering the anchored PKA complex to particular organelles. In most cases, immunochemical and subcellular fractionation techniques have been used to identify AKAPs that are localized to centrosomes (AKAP350) (Keryer *et al.*, 1993), the actin cytoskeleton [Ezrin/AKAP78 (Dransfield *et al.*, 1997a), AKAP250 (Nauert *et al.*, 1997), and AKAP KL (Dong *et al.*, 1998)], the endoplasmic reticulum (AKAP100) (McCartney *et al.*, 1995), the Golgi (AKAP85) Rios *et al.*, 1992), microtubules (MAP2) (Theurkauf and Vallee, 1982), mitochondria (sAKAP84/D-AKAP-1) (Huang *et al.*, 1997; Lin *et al.*, 1995), the nuclear matrix (AKAP95) (Coghlan *et al.*, 1994), the plasma membrane (AKAP15/18 and AKAP79/150) (Bregman *et al.*, 1989; Carr *et al.*, 1992b), and vesicles (AKAP220) (Lester *et al.*, 1996). Less characterized anchoring proteins have been identified in secretory granules, plasma membranes, and the flagella of mammalian sperm (Faux and Scott, 1996a). The subcellular locations of these known AKAPs are indicated in Fig. 6.

More detailed analysis of targeting sequences have been performed on five AKAPs. AKAP220 may be targeted to peroxisomes (Lester *et al.*, 1996), as the last three residues of the protein, Cys-Arg-Leu, conform to a peroxisomal targeting signal 1 (PTS-1) motif that is thought to facilitate the attachment of proteins to the lipid matrix of the peroxisome (Subramani, 1996). AKAP250 is a component of the membrane/cytoskeleton that is enriched in the filopodia of adherent human erythroleukemia cells (Nauert *et al.*, 1997). The amino terminus of AKAP250 contains a consensus myristoylation signal, as well other structural regions that bear some resemblance

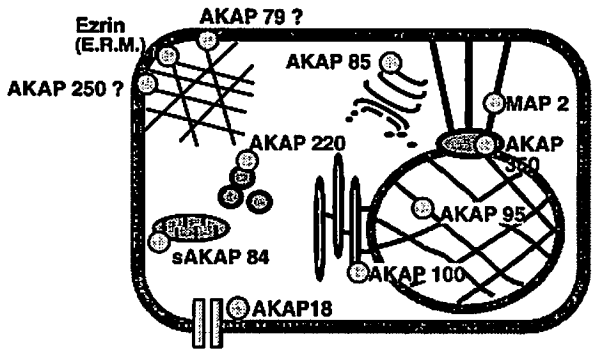

FIGURE 6 Subcellular targeting of AKAPs. A schematic diagram indicating the locations of known AKAPs in a prototypic cell. See text for details.

to actin-binding proteins such as MARCKS and GAP-43 (Aderem, 1992). Likewise, an anchoring protein previously called AKAP78 has now been identified as the cytoskeletal component ezrin (Dransfield *et al.*, 1997a). In fact, ezrin and its two close relatives, radaxin and moesin, bind RII in the overlay assay. All three proteins (E.R.M.) are members of the band 4.1 superfamily of proteins that link the membrane and the cytoskeleton. As will be discussed later, extensive mapping of targeting sequences has been performed on AKAP79 and AKAP18.

1. AKAP79 Targeting

Previous experiments with anchoring inhibitor peptides have demonstrated that AKAP-mediated targeting of PKA is important to regulate excitatory neurotransmitter receptors such as the AMPA and kainate-responsive glutamate receptors (Rosenmund *et al.*, 1994). This function may be fulfilled by AKAP79, a neuronal anchoring protein that has been detected immunochemically in the cell bodies and dendrites of cortical and hippocampal neurons and is enriched in postsynaptic density fractions (Carr *et al.*, 1992b; Glantz *et al.*, 1992; Klauck *et al.*, 1996). AKAP79 also binds the calcium calmodulin-dependent protein phosphatase-2B Calcineurin (CaN) and the calcium and phospholipid-activated protein kinase C (PKC) (Coghlan *et al.*, 1995; Klauck *et al.*, 1996). Hence, it has been proposed that AKAP79 directs the postsynaptic targeting of a multienzyme signaling complex that is involved in coordinating second-messenger-responsive phosphorylation of synaptic proteins (Faux and Scott, 1996b).

The mechanism of AKAP79 targeting to submembrane sites below the plasma membranes and in dendrites has been elucidated (Dell'Acqua *et al.*, 1998). A series of AKAP79 fragments fused to the green fluorescent protein (GFP) were used to define regions in AKAP79 that are sufficient for targeting

(Figs. 7 and 8). Initial experiments confirmed that expression of the full-length AKAP79-GFP fusion in HEK293 cells results in a pattern of membrane localization (Fig. 7C) that is indistinguishable from the localization of the untagged anchoring protein as detected by indirect immunofluorescent staining (Fig. 7B). In contrast, control cells transfected with GFP alone exhibited fluorescence throughout the cytoplasm and the nucleus (Fig. 7A). These results indicate that C-terminal GFP fusion does not adversely affect the subcellular targeting of AKAP79 and confirm the utility of the fluorescent tag approach to map targeting determinants in the anchoring protein.

The N-terminal third of AKAP79 contains three distinct regions of primary structure rich in basic residues: region A, residues 31–52; region B, residues 76–101; and region C, residues 116–145 (Fig. 9A). Previous studies indicated that sequences present in the N-terminal portion of

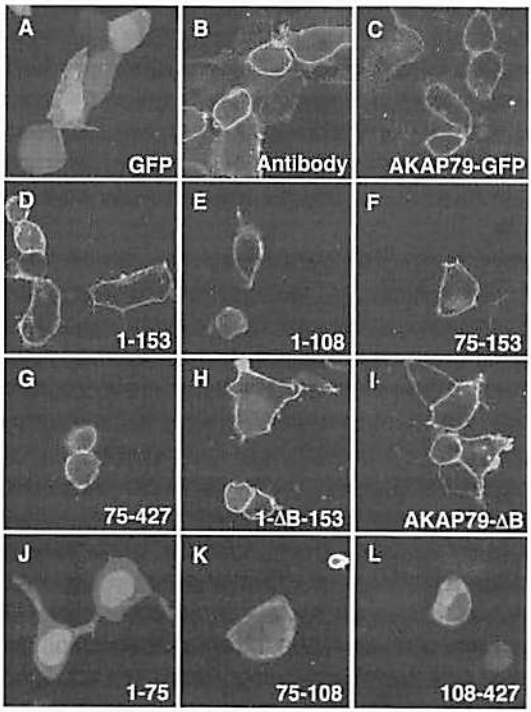

FIGURE 7 Membrane targeting of AKAP79 in HEK-293 cells is mediated by three N-terminal regions. AKAP79-GFP fusion proteins were transiently expressed in HEK-293 cells. Control images for transfections of GFP alone (A) or immunochemical staining (rabbit polyclonal anti-79, FITC) of untagged AKAP79 (B) are also shown. The subcellular localization of each transfected fusion protein (panels C-L) was determined by confocal imaging of GFP fluorescence excited at 490 nm. The images provided correspond to a single confocal plane for a representative field of transfected cells for each fusion construct. The length of each construct is indicated.

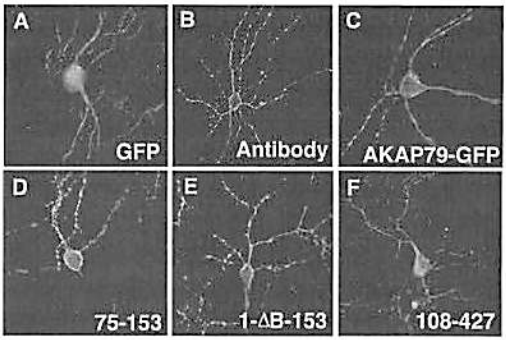

FIGURE 8 Neuronal targeting of AKAP79 is mediated by the N-terminal basic regions. Control neurons expressing GFP alone (A), expressing AKAP-79 GFP (C), or stained immunochemically with rabbit polyclonal anti-150 and FITC-secondary antibodies to visualize endogenous AKAP79/150 (B) are shown. The pattern of cellular GFP fluorescence was imaged in microinjected neurons expressing the 75-153 (D), 1-DB-153 (E), and 108-427 (F) GFP fusion proteins.

AKAP75, termed T1 and T2, which overlap with the A and B basic regions of AKAP79, were involved in targeting in HEK293 cells (Glantz *et al.*, 1993; Li *et al.*, 1996). However, it was previously shown that the A region contains determinants for binding to calmodulin and PKC while other regions interact with CaN (Coghlan *et al.*, 1995; Faux and Scott, 1997; Klauck *et al.*,

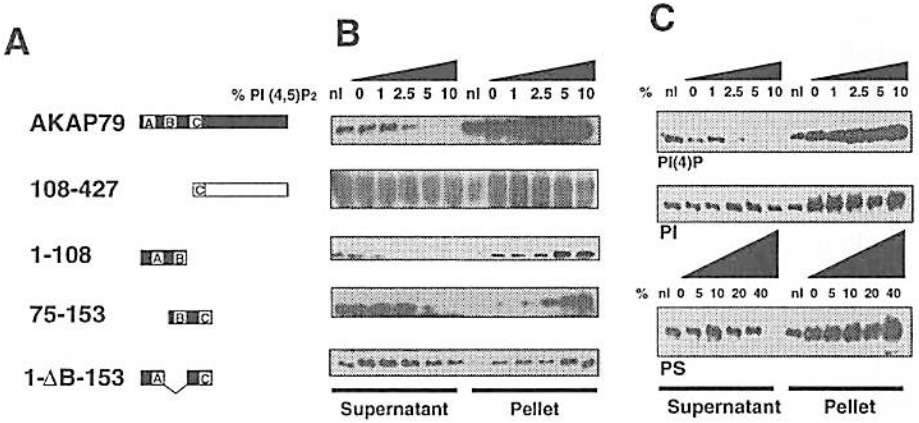

FIGURE 9 AKAP79 basic targeting regions bind to phosphatidylinositol 4,5-bisphosphate. (A) Schematic diagram showing the locations of the A, B, and C membrane targeting regions in AKAP79. (B) A lipid–protein cosedimentation assay was used to detect AKAP79 and fragments that bound to sucrose-loaded phospholipid vesicles. The resulting lipid-bound (pellet) and unbound (supernatant) fractions were detected by immunoblotting with an anti-79 polyclonal antibody. (C) AKAP79 and fragments were analyzed for binding to the vesicles containing the indicated percentages of additional acidic phospholipids: PtdIns(4)P (PI(4)P), PtdIns (PI), and phosphatidylserine (PS).

1996). In order to resolve this issue, AKAP79-GFP fusion proteins containing different combinations of the three basic regions were expressed in HEK293 cells. A fragment encompassing all three basic regions, 1–153, efficiently targeted to the cell periphery, indicating that the N-terminal basic regions alone were sufficient for membrane targeting (Fig. 7D). Likewise, additional constructs containing any combination of two basic regions targeted to the cell periphery (Figs. 7E–7I). At least two of the basic regions were necessary for targeting to the cell periphery, as constructs containing any single basic region failed to effectively target (Figs. 7J–7L). Likewise, microinjection of cDNAs encoding AKAP79-GFP fusion proteins into cortical neurons demonstrated that these basic regions are also responsible for targeting of AKAP79 in neurons (Fig. 8). Collectively, these results suggest that any two of the three AKAP79 basic regions are necessary and sufficient for submembrane targeting in HEK293 cells and cortical neurons.

Although binding determinants for PKC are contained in the A region and those for CaN may overlap with the C region, the results do not support a direct correlation between enzyme binding and membrane targeting (Fig. 9A). Therefore, we concluded that another mechanism must participate in the submembrane targeting of AKAP79. Secondary attachments to the actin cytoskeleton were ruled out, as treatment of HEK293 cells with the actin depolymerizing reagent cytochalasin D did not alter the membrane localization of AKAP79. However, targeting sequences of similar amino acid composition are found in the myristoylated alanine-rich C-kinase substrate protein (MARCKS), GAP43/neuromodulin, and neurogranin. These proteins bind acidic phospholipids such as phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) and calmodulin to regulate their association with the membranes and cytoskeleton (Aderem, 1992; Blackshear, 1993; Houbre *et al.*, 1991; Lu and Chen, 1997). Hence, the phosphoinositide binding activity of purified recombinant AKAP79 and fragments was assessed using a lipid–protein co-sedimentation assay (Mosior and Newton, 1995) (Fig. 9A). AKAP79 bound to sucrose loaded phospholipid vesicles at concentrations of PtdIns(4,5)P_2 as low as 1–2.5% or 1–2.5 μM, suggesting an affinity for PtdIns(4,5)P_2 in the low micromolar range (Fig. 9C). AKAP79 also bound PtdIns(4)P, while no binding was seen for PtdIns (PI) (Fig. 9B), thus suggesting some specificity for recognition of the 4-phosphate on the inositol ring. However, AKAP79 also bound the structurally unrelated acidic phospholipid phosphatidylserine (PS), albeit at much higher concentrations (20–40% or 20–40 μM) (Fig. 9C). Again, mapping experiments suggested that combinations of two or more basic regions are able to mediate AKAP79 binding to acidic phospholipids including PtdIns(4,5)P_2.

In an attempt to elucidate a mechanism for the regulation of AKAP79 targeting, we focused on a potential role for PKA and PKC phosphorylation. AKAP79 is a substrate for PKA and PKC *in vivo*, and most of the potential sites are contained within the first 153 residues of the protein. Although the

A region does not contain any consensus phosphorylation sites, the B and C regions contain several potential sites for PKA or PKC (Faux and Scott, 1997; Klauck *et al.*, 1996) (Fig. 9A). Interestingly, phosphorylation of recombinant AKAP79 fragments encompassing the B and C regions decreased binding to phospholipid vesicles and promoted the release of a fraction of the anchoring protein from cell membranes. Likewise, the presence of 10 μM calmodulin prevented binding of the native protein to phospholipid vesicles. This suggests that Ca^{2+} calmodulin binding as well as protein phosphorylation may regulate AKAP79 targeting.

One conclusion of these studies is that targeting domains of AKAP79 are functionally distinct from the binding sites for RII, PKC, and CaN (Carr *et al.*, 1992b; Coghlan *et al.*, 1995; Klauck *et al.*, 1996) (Fig. 10). This separation of targeting and enzyme binding functions is especially relevant for one of the proposed functions of AKAP79 in neurons, which is to mediate the postsynaptic localization of a kinase/phosphatase signaling complex (Klauck *et al.*, 1996). Localization of such a multienzyme signaling scaffold would allow coordinated regulation of second-messenger-dependent phosphorylation of synaptic substrates such as calcium channels and neurotransmitter receptors (Gao *et al.*, 1997; Rosenmund *et al.*, 1994). In this model the enzymes are bound to the AKAP79 in the inactive state and are then released in response to second messenger stimulation (Fig. 10). For example, active PKA catalytic subunit is released from the PKA holoenzyme by cAMP binding to the AKAP-anchored inhibitory R subunits, while PKC is most

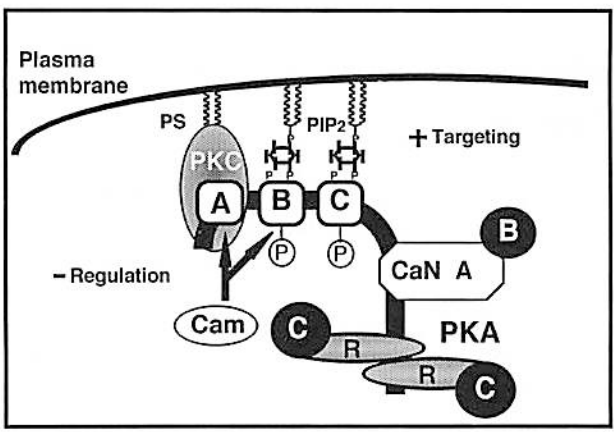

FIGURE 10 A model for regulated membrane targeting of the AKAP79 signaling scaffold. A schematic diagram that indicates the potential domain organization of the membrane targeted AKAP79 signaling complex. Potential positive (+) targeting interactions of the acidic phospholipids, phosphatidylserine (PS) and phosphatidylinositol 4,5-bisphosphate (PIP_2), with PKC and the AKAP79 basic regions (A,B,C) are shown. Possible negative (−) regulation of targeting by protein phosphorylation (PKC and PKA) and calcium signaling (calmodulin) are also depicted.

likely released from inhibition by the anchoring protein in response to intracellular calcium signaling via the competitive binding of calmodulin to AKAP79 (Faux and Scott, 1997).

For the foregoing anchoring model to function properly, it would be important that AKAP79 be targeted at postsynaptic membranes regardless of the docking of individual enzymes to the complex. However, it may be equally important to regulate the AKAP79 targeting interaction. Thus, negative regulatory mechanisms affecting at least two of the three basic domains would be predicted to inhibit membrane binding. This model of regulation (Fig. 10) is consistent with our observations that both protein phosphorylation and Ca^{2+}-calmodulin are able to negatively regulate PtdIns(4,5)P_2 binding for full-length AKAP79 and membrane attachment. These findings are reminiscent of the "electrostatic switch" mechanisms proposed for the reversible translocation of MARCKs protein between the membrane and cytosol in response to PKC phosphorylation or Ca^{2+}-calmodulin (McLaughlin and Aderem, 1995). Hence, regulation of AKAP79 location in response to prolonged second-messenger activation of PKA and PKC or elevation of intracellular calcium could also function as an adaptive negative feedback mechanism that would limit the strength or duration of kinase signaling by repositioning the entire AKAP79 scaffold relative to the locations of substrates or second-messenger generation. Alternatively, phosphorylation or Ca^{2+}-calmodulin mediated redistribution of AKAP79 could act as an amplification mechanism that would remove the inhibitory anchoring protein from close proximity to the released active enzymes, thus favoring maintenance of the activated state. It will be of great interest in the future to further characterize the cellular role of phosphoinositides, protein phosphorylation, and calmodulin in regulation of AKAP79 signaling.

2. *AKAP18 Targeting*

A second membrane associated anchoring protein has been identified that is targeted to the plasma membrane through a different mechanism. A small molecular weight anchoring protein called AKAP15/18 was independently cloned by two groups and has been shown to be functionally coupled to the L-type Ca^{2+} channels (Fraser *et al.*, 1998; Gray *et al.*, 1998). Inspection of the AKAP18 sequence identified three putative signals for lipid modification: a myristoylation site at the N-terminal glycine residue and two palmitoylation sites at cysteines 4 and 5 (Fig. 11A). It was postulated therefore that protein–lipid interactions may promote association of AKAP18 with the plasma membrane. Evidence that these residues undergo lipid modification was derived from subcellular fractionation of HEK293 cells transiently transfected with wild-type AKAP18. Cells fractionated in standard hypotonic buffer show that the heterologously expressed AKAP18 protein segregates exclusively with the particulate fraction (Fig. 11B). However, when cells were fractionated in the presence of increasing concentrations of Triton-

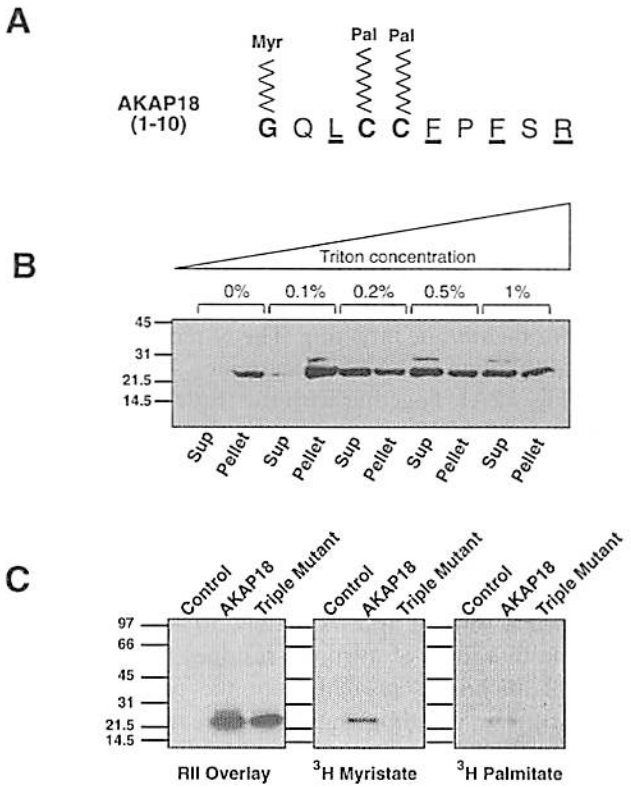

FIGURE 11 AKAP18 is subject to N-terminal lipid modification. (A) The first 10 residues of AKAP18 are shown with the schematic addition of a myristate group on glycine 1 and palmitate groups on cysteines 4 and 5. Hydrophobic residues are underlined. (B) Subcellular fractionation of AKAP18 transfected HEK293 cells in the presence of increasing concentrations of Triton X-100 (0–1%). Soluble (Sup) and particulate (Pellet) fractions at each Triton concentration were separated by SDS–PAGE. AKAP18 protein was detected by immunoblot using a polyclonal antibody raised against recombinant AKAP18. (C) AKAP18 incorporates ^{3}H-myristate and ^{3}H-palmitate in cell culture. HEK293 cells were transfected with wild-type AKAP18 or a mutant with residues Gly^{1}, Cys^{4}, and Cys^{5} substituted with Ala, Ser, and Ser, respectively (Triple mutant) and labeled with ^{3}H-myristate or ^{3}H-palmitate. The presence of AKAP18 protein in immunoprecipitates was confirmed by RII overlay, and ^{3}H incorporation was detected by fluorography.

X 100, 0.2% detergent was sufficient to relocalize a significant proportion of the AKAP18 from the particulate to the soluble fraction (Fig. 11B). Further studies were carried out to determine directly whether myristate and palmitate were incorporated into AKAP18 transiently expressed in culture. HEK293 cells were transfected with wild-type AKAP18 and a mutant in which residues Gly^{1}, Cys^{4}, and Cys^{5} were substituted with Ala, Ser, and Ser, respectively (Triple mutant). The cells were incubated in media containing

^{3}H-myristic acid or ^{3}H-palmitic acid, then subjected to immunoprecipitation with anti-AKAP18 antibodies, and the incorporation of ^{3}H in cell extracts was analyzed by SDS–PAGE and fluorography. Figure 11C shows incorporation of both ^{3}H-myristate and ^{3}H-palmitate by a protein migrating at the correct molecular weight for the wild-type AKAP18 expression construct, whereas there was no significant incorporation of label by the AKAP18 triple mutant. An RII overlay of the same protein samples confirmed the presence of an RII binding protein in both extracts, demonstrating effective expression of both AKAP18 constructs (Fig. 11C). Further mutants were generated to test the hypothesis that residues at the N-terminus of AKAP18 are responsible for membrane targeting. The N-terminal glycine residue was substituted by an alanine (G1A), and cysteines 4 and 5 were changed to serine (C4,5S) (Fig. 12A). Together with the triple mutant described earlier, localization of these AKAP18 mutants was first analyzed by transient expression in HEK293 cells followed by subcellular fractionation. As noted previously, the wild-type AKAP18 protein partitioned exclusively to the particulate fraction (Fig. 12B). Removal of the myristoylation signal alone appeared to have little effect on localization, as the G1A mutant remained exclusively in the particulate fraction (Fig. 12B). In contrast, removal of both palmitoylation signals caused a shift of approximately 50% of the C4,5S mutant to the cytosol, while mutation of all three residues (Triple mutant) caused a complete shift of anchoring protein from the particulate to the soluble fraction (Fig. 12B). These findings supported the notion that lipid modification is involved in the localization of AKAP18 to the plasma membrane.

In order to analyze the subcellular localization of wild-type and mutant AKAP18 proteins inside cells, plasmids were constructed to heterologously express the proteins with a C-terminal GFP fluorescent tag. HEK293 cells transfected with GFP alone exhibited fluorescence throughout the cell, whereas expression of the AKAP18/GFP fusion clearly shows a peripheral staining pattern (Fig. 12C). Control experiments demonstrated that the same peripheral localization was observed in cells transfected with an expression construct encoding the wild-type AKAP18 without a GFP tag (Fig. 12C). In the cell shown, AKAP18 was detected by immunochemical staining with a polyclonal antibody raised against the recombinant protein. These results confirm that AKAP18 is targeted to the cell membrane and that C-terminal fusion of the GFP moiety does not affect the membrane association of the full-length anchoring protein. In contrast, gradual delocalization of AKAP18 was apparent as one (G1A), two (C4,5S), and then three (Triple mutant) lipid modification signals were removed (Fig. 12C).

Our studies show that myristoylation of the N-terminal glycine residue and palmitoylation of cysteines 4 and 5 in AKAP18 are involved in attaching the anchoring protein to the cytoplasmic face of the plasma membrane. Posttranslational modification of proteins by acyl groups is now well established as a mechanism for membrane association of signaling proteins

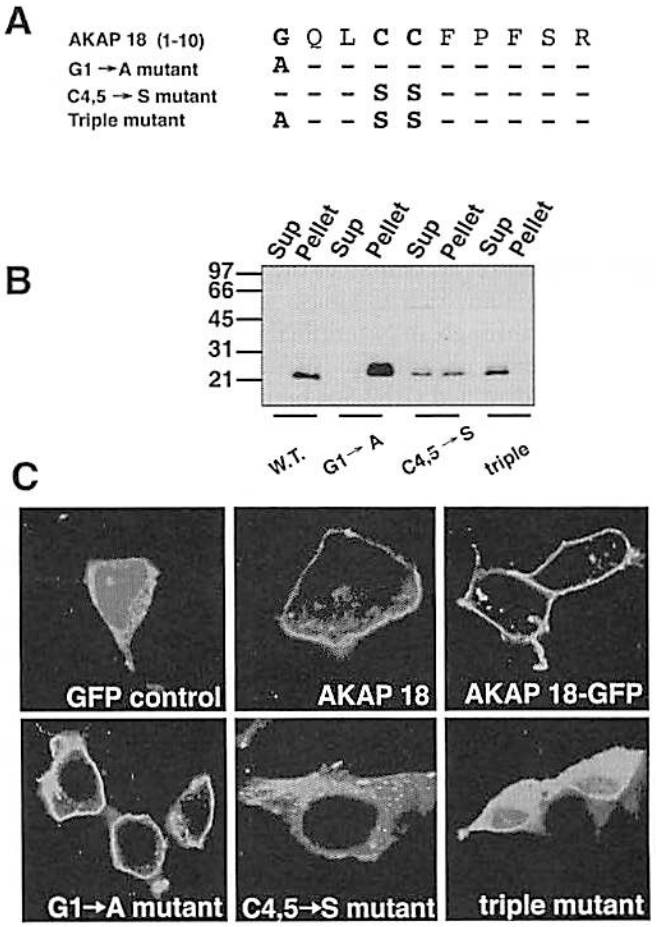

FIGURE 12 Mutations in the first five N-terminal amino acids of AKAP18 disrupt targeting function. (A) Residues 1–10 of AKAP18 showing amino acid changes incorporated for the N-terminal mutants. (B) Subcellular fractionation of HEK293 cells transfected with AKAP18 and the G1A, C4,5S, and triple mutants in the pcDNA3.1/Myc.His expression vector. Soluble (sup) and particulate (pellet) fractions from each construct were separated by SDS–PAGE. AKAP18 proteins were detected by immunoblot using a monoclonal antibody to the c-Myc epitope tag. (C) Confocal microscopy of HEK293 cells transfected with a GFP control plasmid, wild-type AKAP18, and GFP fusions of AKAP18 and the G1A, C4,5S, and triple mutants. The subcellular localization of wild-type AKAP18 was detected by immunochemical staining, whereas the GFP fusions were visualized directly by fluorescent excitation at 490 nm.

(Schlesinger, 1993). One common theme seems to be the presence of multiple sites of lipid modification on the acceptor protein. For example, the Src family of tyrosine kinases contain an N-terminal myristoyl group and one or two palmitoyl groups attached to cysteine residues that contribute to membrane targeting (Kabouridis *et al.*, 1997; Resh, 1994). Likewise, several

of the a subunits of heterotrimeric G-proteins are subject to dual acylation by myristate and palmitate (Milligan *et al.*, 1995). Our studies with point mutations of AKAP18 suggest that any two of the three lipid side chains are sufficient to mediate membrane association. The inability of a single lipid moiety to sustain membrane targeting of AKAP18 is in agreement with reports that one acyl group is insufficient to mediate stable attachment of a protein to a lipid bilayer (Resh, 1994). In fact, membrane-associated proteins that are singly acylated are only able to mediate their attachment when another interaction accompanies the lipid modification. For example, the myristoylated alanine-rich C-kinase substrate protein (MARCKS) is membrane targeted through an N-terminal myristate group and a polybasic region that binds acidic phospholipids (Aderem, 1992; Blackshear, 1993), whereas a recently identified Grb2/Sos binding protein, FRS-2, is myristoylated and has been proposed to bind to the FGF receptor through a PTB domain (Kouhara *et al.*, 1997).

3. *AKAP18 Is Coupled Functionally to the L-Type Ca^{2+} Channel*

It was postulated that membrane targeting of AKAP18 could mediate the localization of PKA in close proximity to transmembrane substrates. Previous studies have suggested that pools of PKA are localized close to skeletal muscle L-type Ca^{2+} channels in order to facilitate rapid and efficient channel phosphorylation (Salvatori *et al.*, 1990). More recent reports have shown that AKAP targeting of the kinase contributes to this process (Burton *et al.*, 1997; Gao *et al.*, 1997; Johnson *et al.*, 1994, 1997). In keeping with this hypothesis, it has been proposed that a low molecular weight AKAP serves to maintain a pool of PKA close to the L-type Ca^{2+} channel (Gray *et al.*, 1997). Given our biochemical evidence that AKAP18 is targeted through protein–lipid interactions to the plasma membrane, we postulated that this anchoring protein may be a physiological partner of the L-type Ca^{2+} channel. A recently established model to test this hypothesis is the reconstitution of PKA modulation of L-type Ca^{2+} channels in HEK293 cells (Gao *et al.*, 1997; Johnson *et al.*, 1997).

Accordingly, whole-cell Ca^{2+} currents were recorded from HEK293 cells transfected with the cardiac α and β Ca^{2+} channel subunits. Okadaic acid (1 μM) was present in the external bath solution and in the pipette solution in order to prevent attenuation of Ca^{2+} current response to cAMP by endogenous phosphatase activity (Gao *et al.*, 1997). Using barium (10 mM) as charge carrier, currents were evoked by depolarization from a holding potential of -80 mV. Whole-cell barium currents activated from -30 mV and peaked at $+10$ to $+20$ mV. Bath application of the cell-permeant cAMP analogue 8-CPTcAMP (1 mM) significantly increased the barium current of cells co-transfected with AKAP18 compared to controls (18.4 $\pm$ 6.5%; $n = 17$ vs 1.1 $\pm$ 2.4%; $n = 12$, $p < 0.05$) (Figs. 13A and 13C). Current augmentation greater than 10% was observed in 9 of 17 cells co-transfected

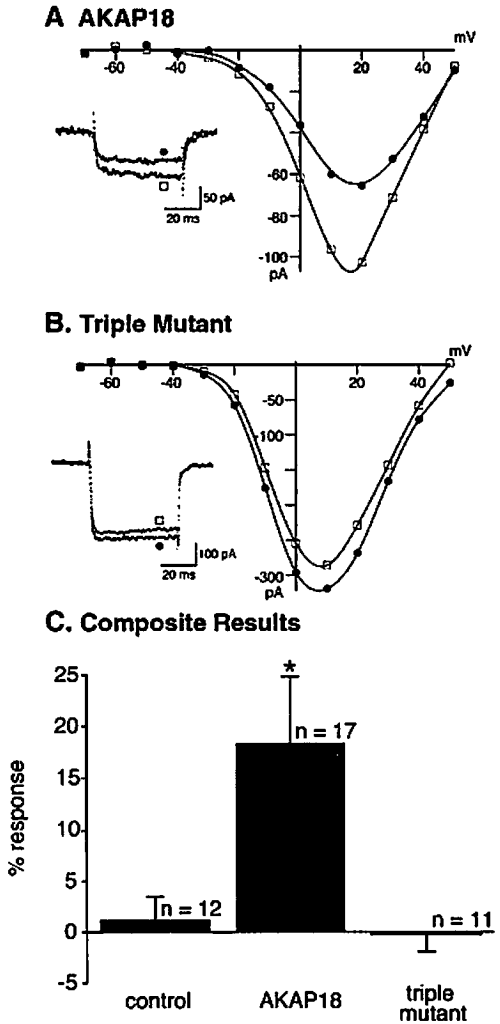

FIGURE 13 Membrane targeting of AKAP18 is required for cAMP-dependent modulation of L-type Ca^{2+} channel currents. (A) Whole-cell current with 10 mM barium as charge carrier was evoked by depolarization from a holding potential of −80 mV. Current–voltage relationship is shown for HEK293 cells transfected with the a_{1c} and b_{2a} cardiac Ca^{2+} channel subunits and wild-type AKAP18. Currents recorded following 2- to 3-min bath application of the cAMP analogue 8-CPTcAMP (1 mM) (open squares) were augmented compared to those before treatment (filled circles). Currents were monitored during drug application by test pulses to 0 mV. Inset shows traces evoked by a voltage step to +10 mV. (B) Modulation of the L-type Ca^{2+} channel was not seen following coexpression of a_{1c} and b_{2a} Ca^{2+} channel subunits with the untargeted AKAP18 triple mutant. Current–voltage relation is shown in the absence (filled circles) and presence (open squares) of 8-CPTcAMP (1 mM). Inset shows currents evoked by a voltage step to 0 mV from a holding potential of −80 mV. (C) Percent response to 8-CPTcAMP for each condition tested. Percent response was calculated as |(8-CPTcAMP−control)/control| × 100%. Response was measured from currents evoked by a voltage step to 0 mV from a holding potential of −80 mV. Barium current through L-type Ca^{2+} channels was significantly ($^*p < 0.05$) augmented by 8-CPTcAMP in AKAP18 transfected cells, while cells transfected with the untargeted triple mutant were not significantly different from controls.

with AKAP18 (34.4 ± 9.3%; $n = 9$), while only 1 of 12 control cells displayed an augmentation. Cells transfected with the triple mutant were not significantly different from controls (−0.3 ± 1.6%; $n = 11$), with 0 of 11 responding positively to 8-CPTcAMP (Figs. 13B and 13C). These results suggest that AKAP18 contributes to the cAMP-dependent augmentation of L-type Ca^{2+} current and that the membrane targeting domain of AKAP18 appears to be required this for modulation.

4. AKAP18 Alters GLP-1 Mediated Insulin Secretion

As previously discussed, it has been shown that subcellular targeting of PKA by AKAPs is required for efficient hormone-mediated insulin secretion in pancreatic beta cells (Lester *et al.*, 1997). Moreover, it has been shown that a significant site of PKA anchoring could be at or proximal to the L-type Ca^{2+} channel, which has previously been implicated a key mediator of the insulin secretion pathway (Bokvist *et al.*, 1995; Gromada *et al.*, 1997; Safayhi *et al.*, 1997; Suga, 1997). Therefore, the effect of AKAP18 on the process of hormone-mediated insulin secretion was measured. A clonal insulin-secreting rat beta cell line, RINm5F, was transfected with plasmids encoding wild-type AKAP18 and the untargeted triple mutant. As expected, immunocytochemical analysis showed that AKAP18 was concentrated at the periphery of the RINm5F cells, whereas the untargeted triple mutant exhibited a more uniform cytoplasmic staining pattern (Fig. 14A). Importantly, co-staining with RII showed that wild-type AKAP18 was able to mediate a redistribution of PKA to the plasma membrane (Fig. 14A). In untransfected cells lacking exogenous AKAP18 staining, RII exhibits a perinuclear staining pattern (Fig. 14A). As no significant AKAP18 staining is observed in these cells, it would appear that RINm5F cells do not contain endogenous AKAP18. The staining pattern for RII was more diffuse in RINm5F cells expressing the untargeted AKAP18 triple mutant (Fig. 14A), in keeping with analogous experiments where PKA anchoring was disrupted using a cytoplasmic RII binding protein (Lester *et al.*, 1997).

To determine if membrane targeting of PKA by AKAP18 influences hormone-mediated signaling events, insulin secretion was measured upon application of the insulinotropic hormone glucagon-like peptide 1 (GLP-1). Insulin secretion from RINm5F cell lines expressing AKAP18 or the untargeted triple mutant was assessed by radioimmunoassay (Drucker *et al.*, 1987; Yaekura *et al.*, 1996). The increase in insulin secretion over basal levels in response to GLP-1 was significantly higher in cells expressing AKAP18 (34.7 ± 8 pmol/min/10^6 cells, $n = 9$) than in pcDNA transfected controls (16.3 ± 2.6 pmol/min/10^6 cells, $n = 6$) (Fig. 14B). Furthermore, expression of the untargeted triple mutant of AKAP18 resulted in a markedly lower level of GLP-1 stimulated secretion relative to controls (10.5 ± 0.5 pmol/min/10^6 cells, $n = 9$) (Fig. 14B). These results suggest that membrane targeting of PKA through its interactions with AKAP18 can facilitate

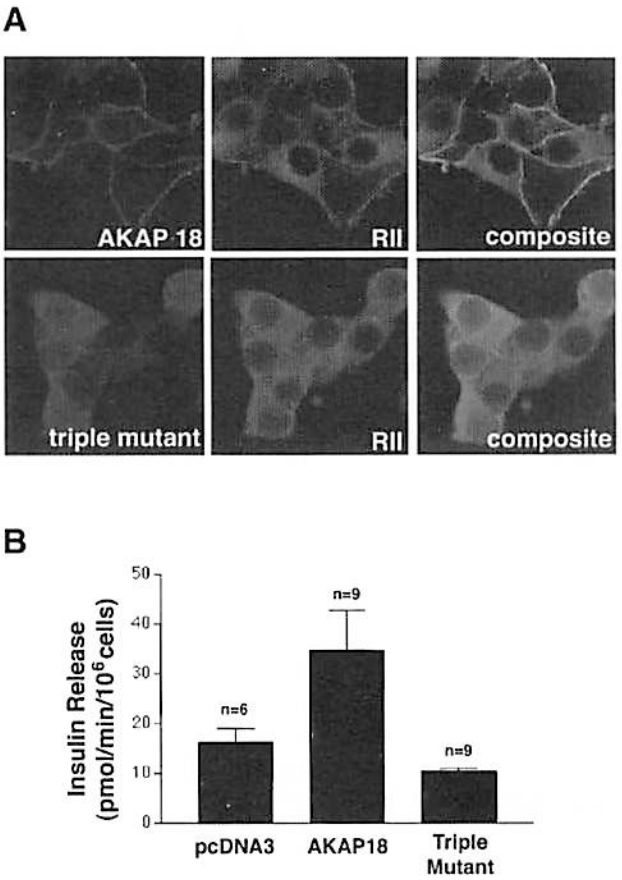

FIGURE 14 AKAP18 mediates a redistribution of RII and influences GLP-1 mediated insulin secretion in RINm5F cells. (A) Confocal microscopy of RINm5F cells transfected with constructs encoding wild-type AKAP18 and the untargeted triple mutant. Immunocytochemical analysis was carried out using polyclonal antibodies raised against recombinant AKAP18 and rat RIIα in rabbit and goat, respectively. Texas Red–conjugated anti-rabbit and FITC-conjugated anti-goat secondary antibodies were used to permit the visualization of both proteins. In the panels showing immunochemical staining of AKAP18 and the triple mutant alone, several untransfected cells are evident because of the nonclonal nature of the AKAP18 transfected RINm5F cell lines. (B) RINm5F cells transfected with wild-type AKAP18, the triple mutant, and a pcDNA3 control plasmid were monitored for GLP-1 mediated insulin secretion. Secretion levels are represented as the increase in pmol insulin released/minute/10^6 cells relative to basal levels.

GLP-1 mediated insulin secretion. This is consistent with the hypothesis that PKA-mediated membrane events, such as phosphorylation of L-type Ca^{2+} channels, are required for hormone-mediated insulin secretion (Bokvist *et al.*, 1995; Gromada *et al.*, 1997; Safayhi *et al.*, 1997; Suga, 1997).

Collectively, these studies suggest that bringing PKA to submembrane sites via association with AKAP18 permits the modulation of L-type Ca^{2+} channels and augments physiological processes that require Ca^{2+} influx, such as hormone-mediated insulin secretion in pancreatic beta cells. However, it must be stressed that a colocalization of PKA with the channel is likely to represent only one of several anchored PKA pools that participate in this process, as multiple AKAPs have been identified in beta cells (L. B. Lester, unpublished observation). PKA substrates involved in the insulin secretion pathway, such as the Glut-2 glucose transporter and the GLP-1 receptor, may have their own pools of anchored PKA (Gromada *et al.*, 1997; Thorens *et al.*, 1996; Widmann *et al.*, 1995, 1996). Nevertheless, these studies provide compelling evidence to support a targeting hypothesis suggesting that second-messenger-mediated signaling events are controlled not only by the catalytic activities of kinases and phosphatases, but also by where these enzymes are localized within the cell (Faux and Scott, 1996b; Hubbard and Cohen, 1993).

IV. Conclusions and Perspectives

In conclusion, we focus on some emerging themes in AKAP research that are likely to yield valuable new information about this family of signaling proteins. Over the past 2 to 3 years there has been a dramatic increase in the cloning of novel AKAPs. This information should provide a more complete database of sequences and establish if the AKAPs merely represent a convergent group of proteins sharing a common RII-binding motif or whether gene families of anchoring proteins exist. Although most of the current data support the first view, it is worth noting that sAKAP84 is expressed in several forms because of alternative splicing, and a novel family of six related anchoring proteins called the AKAP KL family has been reported (Dong *et al.*, 1998). At this time it appears as if some of the sAKAP84 splice variants are differentially targeted to the endoplasmic reticulum and the mitochondria (Chen *et al.*, 1997). Furthermore, one splice variant, called dAKAP1, was isolated in a two-hybrid screen using an RIα fragment as bait (Huang *et al.*, 1997b). Likewise, independent studies have shown that AKAP79 and Ht31 bind RIα, albeit with a 100-fold lower affinity than RIIα (Burton *et al.*, 1997). This raises the intriguing possibility that certain AKAPs are dual-function anchoring proteins which bind both RI and RII. Not only do these findings provide a molecular mechanism for the compartmentalization of RI (Rubin *et al.*, 1972; Skalhegg *et al.*, 1994), but they significantly expand the original anchoring hypothesis to include all PKA holoenzymes. Undoubtedly, future research will discern the nature of the RI–AKAP interactions and establish the physiological significance of type I PKA anchoring.

Another future perspective in our understanding of the AKAPs is likely to come from structural analysis of AKAP–PKA complexes. The solution structure of an RII fragment that encompasses the AKAP-binding site has been solved (Newlon *et al.*, 1997). Modeling studies with the Ht31 peptide suggest that the RII dimer makes multiple contacts with a hydrophobic face of the AKAP peptide. This type of information may explain why the anchoring inhibitor peptides bind RII with such high affinity and are such potent antagonists of PKA anchoring inside cells. No doubt additional studies will focus on structural elucidation of the entire anchored PKA holoenzyme complex.

Another area of future emphasis will be the characterization of AKAP targeting domains. AKAPs such as ezrin, radaxin, moesin, and gravin appear to be anchoring proteins that are linked to both the plasma membrane and the actin cytoskeleton. This suggests that highly localized PKA phosphorylation events may regulate specific signaling events in very defined microenvironments. For example, both AKAP79 and AKAP18 are able to target PKA to the plasma membrane. But are they capable of maintaining distinct localized pools of the kinase in the same cell? These types of macromolecular organization mediated by anchoring proteins not only would place PKA close to certain substrates, but also would cluster the kinase at sites where it could optimally respond to the generation of specific second messengers. The challenge now facing researchers is to pinpoint which important cellular phosphorylation events are regulated by the anchored kinases and phosphatases.

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