with 10 mM tris HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM deoxyribonucleotide triphosphate, 0.05% Triton X-100, 5 μM $(dT)_{24}X$ primer (ATG TCG TCC AGG CCG CTC TGG ACA AAA TAT GAA TTC dT_{24}), and 5 units of Taq polymerase (Perkin-Elmer Cetus, Newton Centre, MA). Cell-free and reverse transcriptase–free samples were used as negative controls. The PCR product was electrophoresed through 1% agarose, stained with ethidium bromide and photographed, transferred to a nylon membrane, and hybridized to the indicated radiolabeled probes with the use of either cDNA clones [human erythropoietin receptor (EpoR), GM-CSFRα, and GM-CSFRβ chains and human c-Kit] or oligodeoxyribonucleotides derived from the cDNA sequence [human c-Mpl (CAGATCAGCTGGGAGGAGCCAAGCACTGAACTTCACCGTCGC), the human IL-1 receptor (IL-1R) (ATAGCAGCCCAGGGCACTTCAGAGTAAGAGGGCTTGGGAAGATCTTTTAAAA), human IL-6R (CTTACTTAGGTGTGGGGGAAGCACCATAACTTTGTTTAGCCCAAAACCAAG), or human gp130 (CTGTACGGCAAGGCGGCTACATGCCTCAGTGAAGGACTAGTAGTT)]. Final wash conditions for the membranes were in 0.2× saline sodium citrate and 0.5% SDS at 40°C.

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Association of Protein Kinase A and Protein Phosphatase 2B with a Common Anchoring Protein

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Specificity of protein kinases and phosphatases may be achieved through compartmentalization with preferred substrates. In neurons, adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase (PKA) is localized at postsynaptic densities by association of its regulatory subunit with an A kinase anchor protein, AKAP79. Interaction cloning experiments demonstrated that AKAP79 also binds protein phosphatase 2B, or calcineurin (CaN). A ternary complex of PKA, AKAP, and CaN was isolated from bovine brain, and colocalization of the kinase and the phosphatase was established in neurites of cultured hippocampal neurons. The putative CaN-binding domain of AKAP79 is similar to that of the immunophilin FKBP-12, and AKAP79 inhibited CaN phosphatase activity. These results suggest that both PKA and CaN are targeted to subcellular sites by association with a common anchor protein and thereby regulate the phosphorylation state of key neuronal substrates.

Protein phosphorylation is a primary means of mediating signal transduction events that control cellular processes. Accordingly, the activities of protein kinases and phosphoprotein phosphatases are highly regulated. One level of regulation is reflected by restriction of the subcellular distribution of several kinases and phosphatases by association with targeting proteins or subunits (*1*), which promotes rapid and preferential modulation of specific targets within a defined microenvironment in response to diffusible second messengers. For example, the type II PKA is targeted by association of its regulatory subunit (RII) with AKAPs (*2*), and disruption of this interaction in neurons affects the modulation of glutamate receptor channels (*3*). In accordance with the targeting subunit hypothesis, AKAPs associate with other cellular components to adapt the kinase for specific roles; however, these additional protein-protein interactions are poorly understood.

To identify AKAP-binding proteins, we used the yeast two-hybrid system (*4*) to isolate complementary DNAs (cDNAs) that encode proteins that associate with AKAP79, a human neuronal anchor protein (*5*, *6*). One positive clone, termed 11.1, contained cDNA for a murine β isoform of the CaN A subunit (*7*). Control experiments with dihybrid crosses showed that CaN specifically interacted with AKAP79; matings of yeast containing CaN cDNA with those expressing Gal4 fusions of RII, casein kinase 1, or phosphodiesterase, or the Gal4 DNA-binding domain alone (pAS1), were negative (Fig. 1). The two-hybrid system also positively identified interactions between RII and itself (dimerization) and between RII and AKAPs 79 or Ht31 (*8*) (Fig. 1). These observations provide evidence for association of AKAP79 with CaN and, because AKAP79 also binds RII, suggest the occurrence of a ternary complex between type II PKA, AKAP79, and CaN.

We used biochemical methods to examine whether PKA and CaN are associated in mammalian brain. Calmodulin-binding proteins were isolated from bovine brain extracts by affinity chromatography, and CaN was immunoprecipitated with affinity-purified antibodies to the CaN A subunit (*9*). Immunoprecipitates were incubated with cAMP, and the resulting eluate was assayed for PKA activity by addition of adenosine triphosphate (ATP) and kemptide substrate. The specific activity of protein kinase was increased by purification of a 30 to 60% saturated ammonium sulfate fraction of the extract on calmodulin-agarose (28 ± 6-fold; mean ± SD, $n = 3$) and by immunoprecipitation with specific antibodies to CaN (123 ± 3.6-fold) (Fig. 2A).

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All of the protein kinase activity in the immunoprecipitate was inhibited by PKA inhibitor peptide (PKI), a specific inhibitor of PKA, indicating that the catalytic subunit of PKA was a component of the isolated complex. If the catalytic subunit associates with CaN by means of AKAP75 (the bovine homolog of AKAP79) (*10*), then both RII and AKAP75 should also be present in the precipitated complex. Indeed, proteins corresponding in size to RII and AKAP75 (both substrates for the catalytic subunit) were present in the immunoprecipitate and were phosphorylated on addition of cAMP and [γ-^{32}P]ATP (Fig. 2B). Coimmunoprecipitation of AKAP75 was confirmed by analysis of protein blots with a ^{32}P-labeled RII overlay method (Fig. 2C) (*11*). In complementary experiments, R subunits of PKA were isolated from extracts of bovine brain by affinity chromatography on cAMP-agarose (9). A proportion of the CaN present in the extract copurified with R subunits and was eluted from the affinity matrix with cAMP (Fig. 2, D and E). Because recombinant CaN did not bind to cAMP-agarose, the phosphatase must have been purified as part of a ternary complex with the AKAP and R subunit. Analysis by RII overlay showed that AKAP75 was also present in the fraction eluted from the cAMP-agarose by cAMP (Fig. 2F). The binding of CaN to cAMP-agarose was mediated by AKAP75: Specific displacement of AKAP75 from RII bound to the affinity column with a PKA anchoring inhibitor peptide, Ht31(493–515) (*12*), also eluted CaN (Fig. 2, G and H), whereas RII remained associated with the affinity column and was subsequently eluted with cAMP (Fig. 2I). These results demonstrate simultaneous association of CaN and PKA with the AKAP. Formation of this ternary complex may account for the previous observation that CaN copurifies with PKA and an unidentified 75-kD protein (*13*). From the amounts of protein recovered, we estimate that ~5% of the total CaN present in the brain lysate was purified with the AKAP75-RII complex (*14*). Given that CaN is abundant in brain, representing up to 1% of total protein (*15*), the fraction associated with the AKAP and PKA should represent a significant amount of phosphatase activity. However, we did not detect phosphatase activity in the complexes eluted from cAMP-agarose with either cAMP or the Ht31(493–515) peptide, indicating that the purified CaN was destabilized or otherwise inactivated (*16*).

The binding of both PKA and CaN to AKAP79 implies that the AKAP contains distinct sites for kinase and phosphatase binding. Residues 388 to 409 of AKAP79 represent the PKA-binding site (*5*, *12*). Residues 88 to 102 of AKAP79 were con-

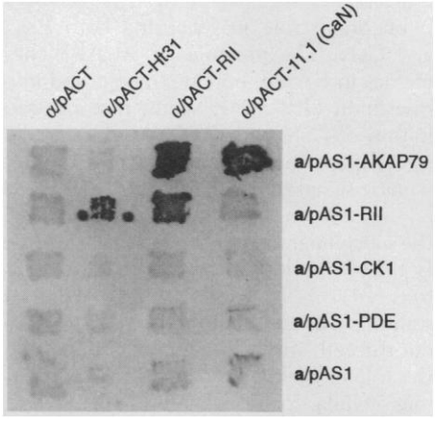

Fig. 1. Interaction of CaN with AKAP79 in yeast. Complementary DNA encoding CaN A_β (amino acids 10 to 525) was obtained by screening a cDNA library with the yeast two-hybrid system (*4*) in an attempt to identify clones encoding proteins that interact with AKAP79. Test crosses are shown for yeast dihybrid matings (*MAT*α × *MAT***a**) in which association of expressed proteins results in the expression of a β-galactosidase reporter gene (indicated in black). Interactions of AKAP79 with either RII or CaN were identified as positive. Also identified were control interactions of RII with itself (dimerization) and between RII and the human thyroid AKAP Ht31 (*12*). CK1, casein kinase 1; PDE, phosphodiesterase.

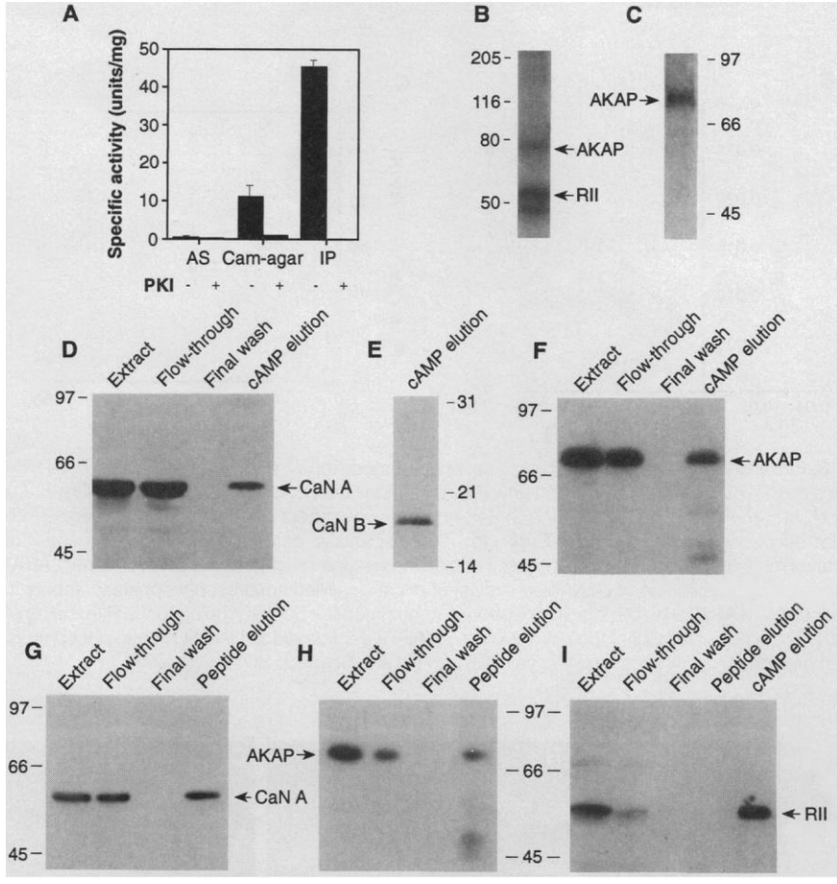

Fig. 2. Copurification of CaN and PKA from brain. (**A** to **C**) CaN was purified from bovine brain by affinity chromatography with calmodulin-agarose and immunoprecipitation with affinity-purified antibodies to CaN A (*9*). (A) Specific activity of kinase in the 30 to 60% ammonium sulfate fraction (AS), after elution from calmodulin-agarose (Cam-agar), and after elution of the immunoprecipitate with cAMP (IP); activity was measured in the presence (+) or absence (−) of PKA PKI. Values are means ± SD. (B) Autoradiograph of an SDS polyacrylamide gel showing proteins phosphorylated in the immunoprecipitate by endogenous PKA. The migration positions of molecular size markers (in kilodaltons) and of phosphorylated RII and AKAP75 are indicated. (C) ^{32}P-RII overlay detection of AKAP75 in the immunoprecipitate. (**D** to **I**) In complementary experiments, R subunits of PKA were purified from crude extracts of bovine brain by affinity chromatography with cAMP-agarose. Protein immunoblots were probed with affinity-purified antibodies to the CaN A subunit (D), with a monoclonal antibody (UBI) to the CaN B subunit (E), or with ^{32}P-labeled RIIα to detect AKAPs (F). Similar experiments were performed with Ht31(493–515) peptide to specifically displace AKAPs from the cAMP affinity column. Protein blots were probed with antibodies to CaN A (G), ^{32}P-RII (H), or antibodies to RII (I). Positions of molecular size markers are indicated in kilodaltons. Extract and flow-through lanes each contained 50 μg of protein. All other lanes (including peptide elution) contained 25 ng of protein, except cAMP elution lanes in (D) through (F), which contained 25 μg of total protein (mostly R subunit).

sidered likely to constitute the CaN-binding site because of similarity to a region of the immunophilin FKBP-12 that contains determinants for CaN association (Fig. 3A) (*17*). Binding of FKBP-12 to CaN is dependent on the immunosuppressant drug FK506 and results in inhibition of the phosphatase (*18*). Given that CaN was inactive when isolated as a complex with the AKAP, we examined the effects of recombinant AKAP79 on CaN phosphatase activity (*19*). AKAP79 inhibited the activity of both full-length CaN (which is dependent on Ca^{2+} and calmodulin) and a truncated, constitutively active form of CaN, CaN_{420} (which is not dependent on Ca^{2+} and calmodulin), in a noncompetitive manner [inhibition constant (K_i), 4.2 ± 18 μM; n = 3) with respect to the phosphorylated RII substrate peptide (Fig. 3B) (*20*). Furthermore, a synthetic peptide corresponding to AKAP79 residues 81 to 102 inhibited both forms of CaN, whereas the Ht31(493–515) peptide did not inhibit CaN (Fig. 3C). The observed inhibition by the AKAP79 peptide was specific for calcineurin; the peptide did not affect the activity of protein phosphatases 1 or 2A at peptide concentrations as high as 0.4 mM. Although the CaN-binding sites on AKAP79 and FKBP-12 are similar, their differences may have functional relevance: FK506 (2 μM) did not affect the potency of inhibition by AKAP79, and recombinant AKAP79 did not display petidyl prolyl isomerase activity toward a fluorescent peptide substrate (*21*, *22*). These data suggest that CaN is localized by AKAP79 in its inactive state in a manner analogous to that of the PKA holoenzyme. Whereas PKA is activated as a result of interaction with cAMP, regulation of the association of CaN with AKAP79 is likely to involve multiple factors. Our observation that AKAP79 inhibited the phosphatase both in the absence and presence of both Ca^{2+} and calmodulin is not consistent with a simple model in which the binding of Ca^{2+} and calmodulin dissociates CaN from the AKAP.

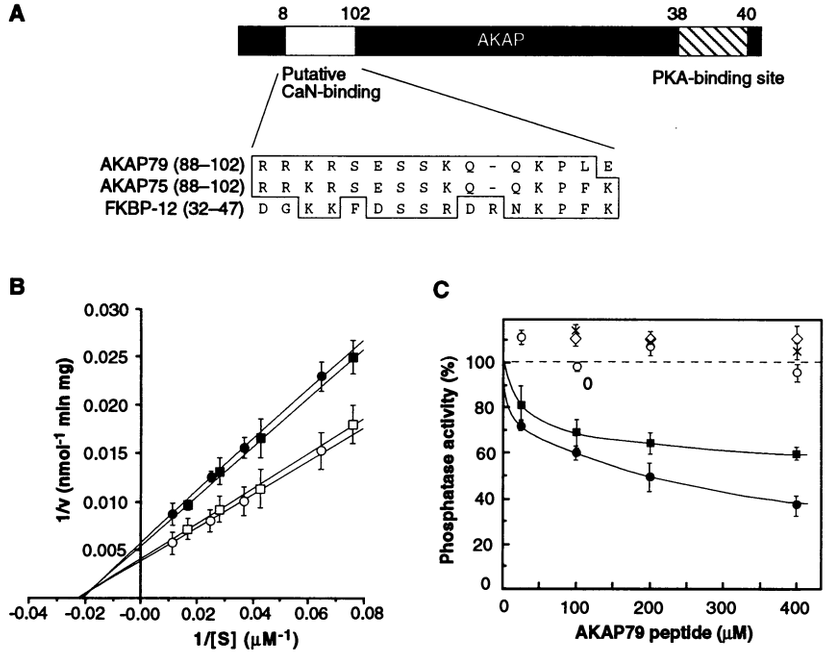

Fig. 3. Inhibition of CaN by AKAP79. (**A**) Sequence comparison of sites within AKAP79, AKAP75, and bovine FKBP-12. Abbreviations for the amino acid residues are D, Asp; E, Glu; F, Phe; G, Gly; K, Lys; L, Leu; N, Asn; P, Pro; Q, Gln; R, Arg; and S, Ser. Amino acid numbers are indicated. Boxed areas indicate similar sequences. (**B**) Lineweaver-Burk plot of phosphatase activity of CaN (circles) and CaN_{420} (squares) measured in the absence (open symbols) or presence (filled symbols) of recombinant AKAP79. (**C**) Dose-response curves of CaN (filled circles) and CaN_{420} (filled squares) phosphatase activity in the presence of AKAP79(81–102) inhibitor peptide. A control peptide corresponding to the RII-binding site of Ht31 (*12*) did not inhibit CaN (open circles). The AKAP79 peptide did not inhibit protein phosphatases 1 (open diamonds) or 2A (crosses). Values in (B) and (C) are means ± SD.

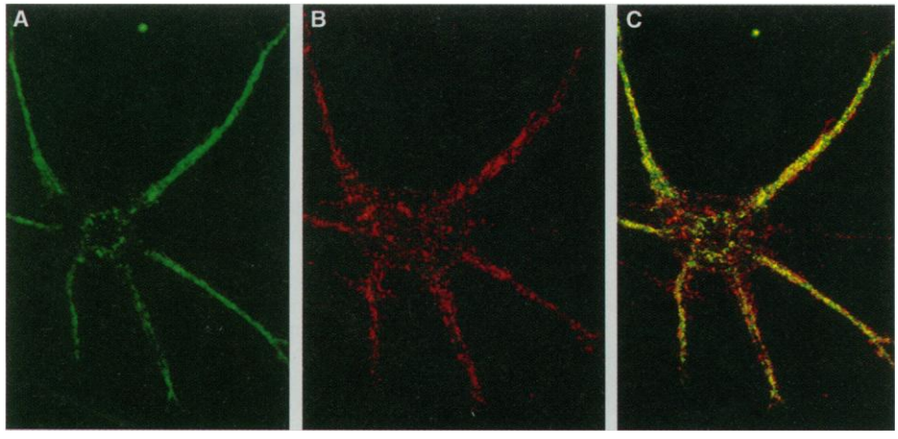

Fig. 4. Colocalization of RII and CaN. Indirect immunofluorescence was used to detect RII and CaN in cultured rat hippocampal neurons. (**A**) Staining pattern for RII (green) obtained with affinity-purified antibodies to RII (1:1000 dilution). (**B**) Staining pattern for CaN (red) in the same cell with antibodies specific for the CaN A subunit (1:1000 dilution). (**C**) Double-staining for CaN and RII displayed by superimposing the images shown in (A) and (B). Magnification, ×500.

The subcellular location of many protein kinases and protein phosphatases is defined by association with targeting subunits (*1*). Our results show that AKAP79 serves a bifunctional role in localizing both PKA and CaN. The presence of AKAP79 homologs in bovine, porcine, rabbit, and murine brain (*10*, *23*) indicates that colocalization of CaN with PKA may be a widespread phenomenon that adapts neurons for specific signal transduction events. We used immunocytochemical methods to examine the subcellular distribution of CaN and type II PKA in cultured rat hippocampal neurons (*24*). CaN (Fig. 4A) and RII (Fig. 4B) staining was regionally dispersed throughout the cell, an observation consistent with the role of these abundant proteins in various cellular processes (*25*). There were, however, distinct regions where the distribution of RII and CaN overlapped, and these regions were primarily confined to neurites (Fig. 4C). These observations are consistent with colocalization of limited amounts of CaN and type II PKA by the AKAP, and they suggest a role for the ternary complex in regulating synaptic transmission. This hypothesis is supported by studies showing that RII and AKAP colocalize in neurites (*3*) and that AKAP79 (*5*), type II PKA (*26*), and CaN (*27*) are components of postsynaptic densities. Potential substrates for the localized ternary

transduction complex are likely to include synaptic receptor-channel proteins, such as α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA)–kainate–sensitive glutamate receptors, which are modulated by AKAP-targeted PKA (*3*).

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24. Cells were cultured, fixed with formalin, and immunostained as described (*3*). Affinity-purified goat antibodies to RII and fluorescein isothiocyanate–conjugated donkey antiserum to goat IgG were used for RII staining; affinity-purified rabbit antibodies to CaN, biotinylated donkey antibodies to rabbit IgG, and streptavidin–Texas red (Jackson) were used for CaN staining. Images were obtained with a Bio-Rad MRC-600 confocal laser scanning system (A1 and A2 filters) with a Nikon Optiphot 2 microscope equipped with 60× planappo chromat (numerical apperture, 1.6) oil immersion lens. Confocal sections had an absolute thickness of between 1.5 and 2 μm. Specificity of immunostaining was confirmed by the absence of fluorescence in cultures incubated with secondary antibodies alone and in experiments with preimmune sera.
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