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cAMP Signaling in Neurons: Patterns of Neuronal Expression and Intracellular Localization for a Novel Protein, AKAP 150, that Anchors the Regulatory Subunit of cAMP-Dependent Protein Kinase IIβ

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In mammalian brain, physiological signals carried by cyclic AMP (cAMP) seem to be targeted to effector sites via the tethering of cAMP-dependent protein kinase IIβ (PKAIIβ) to intracellular structures. Recently characterized A kinase anchor proteins (AKAPs) are probable mediators of the sequestration of PKAIIβ because they contain a high-affinity binding site for the regulatory subunit (RIIβ) of the kinase and a distinct intracellular targeting domain. To establish a cellular basis for this targeting mechanism, we have employed immunocytochemistry to 1) identify the types of neurons that are enriched in AKAPs, 2) determine the primary intracellular location of the anchor protein, and 3) demonstrate that an AKAP and RIIβ are coenriched and colocalized in neurons that utilize the adenylate cyclase-cyclic AMP-dependent protein kinase (PKA) signaling pathway. Antibodies directed against rat brain AKAP 150 were used to elucidate the regional, cellular and intracellular distribution of a prototypic anchor protein in the CNS. AKAP 150 is abundant in Purkinje cells and in neurons of the olfactory bulb, basal ganglia, cerebral cortex, and other forebrain regions. In contrast, little AKAP 150 is detected in neurons of the thalamus, hypothalamus, midbrain, and hindbrain. A high proportion of total AKAP 150 is concentrated in primary branches of dendrites, where it is associated with microtubules. We also discovered that the patterns of accumulation and localization of RIIβ (and PKAIIβ) in brain are similar to those of AKAP 150. The results suggest that bifunctional AKAP 150 tethers PKAIIβ to the dendritic cytoskeleton, thereby creating a discrete target site for the reception and propagation of signals carried by cAMP.

INTRODUCTION

Cyclic AMP-dependent protein kinase IIβ (PKAIIβ)[1] is the predominant cyclic AMP-dependent protein kinase (PKA) isoform and principal mediator of cyclic AMP (cAMP) action in the mammalian central nervous system (CNS) (reviewed in Bregman *et al.*, 1989, 1991; Scott, 1991). Approximately 70% of PKAIIβ is associated with the particulate fraction of brain homogenates (Stein *et al.*, 1987). In contrast, nearly all of the PKA activity in other tissues partitions with the cytosol (Beebe and Corbin, 1986; Edelman *et al.*, 1987). The tethering of brain PKAIIβ is due, in part, to the high affinity binding of the regulatory subunit (RIIβ) of the enzyme with particulate A kinase anchor proteins (AKAPs) (Sarkar *et al.*, 1984; Bregman *et al.*, 1989, 1991). The formation of stable AKAP-RIIβ complexes provides a plausible mechanism for targeting signals carried by cAMP to specific effector sites within brain cells. AKAP-RIIβ complexes could also place PKAIIβ in proximity with its physiological substrates, thereby ensuring rapid and efficient signal transmission.

The AKAPs constitute a unique family of proteins that are expressed principally in brain (Bregman *et al.*,

[1] Abbreviations used: AKAP, A kinase anchor protein; MAP2, microtubule-associated protein 2; PKA, cyclic AMP-dependent protein kinase; PKAIIβ, the type IIβ isoform of cyclic AMP-dependent protein kinase; RIIβ and RIIα, the regulatory subunit isoforms of cAMP-dependent protein kinases IIβ and IIα, respectively.

1989, 1991). Complementary DNAs encoding AKAPs from bovine (AKAP 75), human (AKAP 79), and rat (AKAP 150) brain have been cloned and characterized (Bregman *et al.*, 1989, 1991; Hirsch *et al.*, 1992). AKAP 75, which is encoded by an intron-less gene (Hirsch *et al.*, 1992), has been studied as a prototypic anchor protein. It contains 428 amino acid residues and is unrelated to all previously studied proteins except AKAP 150 and AKAP 79. The positively charged, N terminal region of AKAP 75 is essential for its intracellular anchoring function, whereas a segment of the polypeptide located near the C terminus contains a high-affinity binding site for RIIβ (Bregman *et al.*, 1991; Hirsch *et al.*, 1992). AKAP 150 and AKAP 79 have C terminal, RIIβ binding domains that are 90% identical with residues 333–416 in AKAP 75 (Hirsch *et al.*, 1992).

Despite the progress made in defining the molecular and biochemical properties of AKAPs many fundamental questions remain regarding the roles of AKAPs at the levels of integrated brain tissue, individual cells, and subcellular compartments. For example: What is the regional distribution of AKAPs in brain? Are AKAPs evident only in certain brain cells? Which cells are highly enriched with AKAPs? What are the intracellular structures to which AKAPs and PKAIIβ (via RIIβ) are bound? Are the locations of AKAP and RIIβ in intact brain consistent with the idea that anchor proteins mediate the targeting of signals generated in response to neurotransmitters that increase the concentration of cAMP in neurons?

To address the preceding questions we have now determined the regional, cellular, and intracellular distributions of AKAP 150 and RIIβ in rat brain by systematic immunocytochemical analysis.

MATERIALS AND METHODS

Antibodies

A polyclonal antiserum directed against the 256-residue C terminal segment of bovine AKAP 75 was produced in rabbits as previously described (Bregman *et al.*, 1991). The immunoglobulin Gs (IgGs) in this serum bind cytosolic, detergent-solubilized, and nitrocellulose-immobilized rat brain AKAP 150 with high affinity and specificity. Proteins other than AKAP 150 are not detected when Western blots of total cytosolic and particulate fractions of rat brain are probed with these antibodies (see Figure 1). Details regarding the preparation, characterization, and cross-species reactivity of the serum are presented in previous publications (Bregman *et al.*, 1989, 1991). Preimmune serum from the producer rabbit was used as a control for immunocytochemical analysis. This reagent exhibited no reactivity with AKAP 150, RIIβ, or other proteins.

A polyclonal rabbit antiserum directed against bovine brain RIIβ was prepared as previously described (Erlichman *et al.*, 1980). This antiserum binds RIIβ with a 20-fold higher affinity than RIIα (Erlichman *et al.*, 1980; Stein *et al.*, 1987). IgGs were purified from the serum by protein A affinity chromatography (Ey *et al.*, 1978). Next, anti-RII IgGs that bound epitopes shared by RIIβ and RIIα were removed by incubation (4 d at 4°C) with excess purified bovine heart RIIα (Erlichman *et al.*, 1980) that was immobilized on an Immobilon-P membrane. Before adsorption, the membrane containing RIIα was blocked with 10 mM sodium phosphate, pH 7.4/0.15 M NaCl (phosphate-buffered saline [PBS]) containing 5% (wt/vol) albumin for 1 h at 37°C. The monospecificity of the remaining IgGs was confirmed by probing a Western blot containing samples of 1) purified bovine brain RIIβ, 2) purified bovine heart RIIα, 3) rat neocortex homogenate, and 4) purified recombinant RIIβ (see Figure 1). Nonimmune serum and purified nonimmune rabbit IgGs were utilized as controls for immunocytochemistry and yielded similar negative results.

Preparation of Cytosolic and Particulate Fractions from Rat Brain and Pituitary

Samples of neocortex, olfactory bulb, caudate-putamen, hippocampus, brain stem, cerebellum, spinal cord, and pituitary were isolated from male rats by dissection. The various tissue samples were suspended (1:5, wt/vol) in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-NaOH buffer, pH 7.4, containing 20 mM NaCl, 2 mM dithiothreitol, 5 mM EDTA, 2 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 10 μg/ml leupeptin, 5 μg/ml aprotinin, 5 μg/ml pepstatin, 50 μg/ml chymostatin, 25 μg/ml antipain, 10 mM benzamidine-HCl, 50 μg/ml soybean trypsin inhibitor, 10 μM (transepoxysuccinyl)-L-leucylamido (4-guanidino) butane, 0.1 μg/ml bestatin, 1 μg/ml phosphoramidon, and 50 μg/ml (4-amidinophenyl) methanesulfonyl fluoride (buffer A) and were disrupted by using a Teflon-glass homogenizer with a motor-driven pestle. The resulting homogenates were fractionated by centrifugation at 150,000 $\times$ *g* for 45 min. The cytosol (supernatant solution) was collected and the particulate fraction (pellet) was resuspended in buffer A (3.5 ml/0.1 g of tissue in the original homogenate). All operations were performed at 0–4°C. Protein concentrations were determined by the procedure of Bensadoun and Weinstein (1976) with bovine serum albumin as a standard.

Western Immunoblot Analysis

Proteins from the various CNS regions and pituitary were fractionated in a 9% polyacrylamide gel containing 0.1% sodium dodecyl sulfate and then transferred to an Immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) as previously described (Bregman *et al.*, 1989). To ensure the efficient transfer of AKAP 150 to the membrane, electroblotting was performed for 36 h at a constant current of 0.1 A (25 V). Nonspecific binding sites were blocked by incubating the membranes in buffer B (10 mM sodium phosphate, pH 7.4 containing 0.15 M NaCl, 5% (wt/vol) Carnation nonfat dry milk, 0.1% (wt/vol) bovine serum albumin and 0.02% NaN_3) (6 ml/lane) for 1 h at 37°C. Next, the filters were incubated with anti-AKAP 150 serum (1:4000) in buffer B (4 ml/lane) for 90 min at 22°C. Subsequently the filters were washed serially for 10 min with PBS, 15 min with PBS containing 0.1% (vol/vol) NP-40, and 10 min with PBS, using 6 ml of buffer per lane. Antigen-antibody complexes were radiolabeled by incubating the PVDF membranes with buffer B (4 ml/lane) containing ^{125}I-protein A (2×10^5 cpm/ml) prepared by the method of Schubart and Fields (1984). Finally, the blots were washed as described above, air-dried, and exposed to XAR-5 X-ray film (Kodak, Rochester, NY) at −75°C to generate autoradiographic signals.

A similar protocol was used to detect RIIβ subunits. However, buffer B was replaced with Tris-buffered saline {TBS; 10 mM tris(hydroxymethyl)aminomethane [Tris]-HCl, pH 7.4, containing 0.15 M NaCl} supplemented with 5% (wt/vol) albumin and the washes were performed with TBS. Anti-RIIβ IgGs were used at a final concentration of 1.5 μg/ml.

Assay for RIIβ Binding Activity

The methodology and application of the overlay binding assays have been described in several published papers (Leiser *et al.*, 1986; Bregman *et al.*, 1989, 1991). In brief, a Western blot is probed with ^{32}P-labeled RIIβ (with an RIIβ concentration of 0.3 nM and 1×10^5 cpm ^{32}P-radioactivity/ml) and the RIIβ binding protein (AKAP 150) is visualized by autoradiography.

Immunocytochemistry and Microscopy

Twelve rats were used for replicate determinations of the regional and cellular distribution of AKAP 150 in brain. Consecutive serial sections from eight of these brains were used to elucidate the localization of RIIβ. Male rats (350 g) were anesthetized with sodium pentobarbital (45 mg/kg ip) and perfused transcardially with PBS for 4 min. Subsequently, the perfusion was continued with 4% paraformaldehyde in PBS for 30 min (~20 ml/min). After terminating the perfusion, the brains were removed and postfixed in 4% paraformaldehyde for 16 h at 4°C.

Coronal sections (50 μm) were cut by a vibratome, collected by flotation in PBS, and probed with anti-AKAP 150 or anti-RIIβ antibodies in suspension in 12-well cell culture dishes. The avidin/biotin immunoperoxidase staining method of Hsu *et al.* (1981) was employed to visualize the antigens. Incubations were performed at room temperature unless indicated otherwise. Sections were permeabilized with 0.15% (vol/vol) Triton X-100 in PBS for 60 min. Endogenous peroxidase activity was quenched by incubating the samples with 0.5% H_2O_2 in PBS for 20 min. Nonspecific binding sites were blocked by incubation with 10% (vol/vol) normal goat serum in PBS for 60 min. Consecutive tissue sections were then incubated for 16 h at 4°C with either anti-AKAP 150 serum (1:4000), preimmune serum (1:4000), anti-RIIβ IgG (0.7 μg/ml) or nonimmune rabbit IgG (0.7 μg/ml) diluted in PBS containing 2% goat serum. Subsequently, the sections were incubated sequentially with biotinylated goat anti-rabbit IgG (1:200 in PBS-2% goat serum) for 1 h and 1% avidin complexed with biotinylated horseradish peroxidase (ABC complex; Vector Laboratories, Burlingame, CA) in PBS for 1 h. Finally, the sections were incubated with the peroxidase substrate diaminobenzidine (0.05%) and 0.01% H_2O_2 in 50 mM Tris-HCl pH 7.6, for 8 min. Each of the incubations described above was terminated by washing four times with PBS (10 min each). Immunostained sections were mounted on slides in aqueous mounting medium (Gel/Mount) and examined with a Nikon Labphot microscope (Garden City, NY). Photographs were taken with Ilford PanF (Essex, UK) or Kodak T-Max film.

Each brain region described under RESULTS was independently sampled and analyzed by immunoperoxidase staining in five separate experiments. The results were essentially the same in each instance.

For electron microscopy, rats were perfused with 3% paraformaldehyde and 0.25% glutaraldehyde in PBS, pH 7.4. Brains were postfixed for 1 h in the same solution. Vibratome sections were incubated in 0.5% $NaBH_4$ for 30 min to eliminate residual aldehydes. Permeabilization, blocking, and immunoperoxidase staining were performed as described above. Vibratome sections immunostained with antibodies against AKAP 150 or preimmune serum were postfixed for 1 h in 1% OsO_4 in 0.1 M sodium cacodylate buffer, pH 7.4, rinsed in H_2O and stained with 1% uranyl acetate. After dehydration with a graded series of ethanol solutions and propylene oxide, the sections were embedded in LX112 Epon resin. Thin sections (0.06 μm) were cut on an ultramicrotome and examined in a JEOL (Peabody, MA) JEM-1200 EX electron microscope. Thin sections were not stained with uranyl and lead salts to obtain optimal resolution of the peroxidase reaction product.

Materials

Fraction V bovine serum albumin was obtained from Miles Laboratories (West Haven, CT). Protease inhibitors were acquired from Sigma (St. Louis, MO) and Boehringer Mannheim (Indianapolis, IN). $Na^{125}I$ and [γ-^{32}P]ATP were purchased from Amersham (Arlington Heights, IL). Biotinylated goat anti-rabbit IgG and avidin-complexed biotinylated horseradish peroxidase H were obtained from Vector Laboratories. Normal goat serum and normal rabbit IgGs were from GIBCO (Grand Island, NY) and Cappel Laboratories (Duram, NC), respectively. Paraformaldehyde, glutaraldehyde, hydrogen peroxide, and diaminobenzidine were obtained from Sigma. Sprague-Dawley rats were acquired from Taconic Farms (Germantown, NY).

RESULTS

Characterization of Anti-AKAP 150 and Anti-RIIβ Antibodies

Experiments documenting the efficacy, potency, and specificity of anti-AKAP 150/AKAP 75 antibodies were reported in several recent papers (Bregman *et al.*, 1989, 1991). Further evidence for their high affinity and specificity for AKAP 150 in rat brain is presented in Figure 1C.

Anti-RIIβ IgGs bound both RIIβ purified from brain and homogenous recombinant RIIβ but were unable to complex RIIα (Figure 1, A and B). These monospecific IgGs also avidly and selectively bound the RIIβ subunit in a homogenate of rat cerebral cortex and in the total particulate fractions derived from other regions of rat brain (Figure 1, A, B, and D). The binding of anti-RIIβ IgGs to their target antigen in Western blots was inhibited when the antibodies were preincubated with an excess of native or recombinant RIIβ. Thus the antibodies used in the following studies have the necessary selectivity to determine the distributions of AKAP 150 and RIIβ in the CNS.

Expression of AKAP 150 and RIIβ in Dissected Regions of the CNS

The abundance of AKAP 150 varied markedly in different regions of the rat CNS. Both the Western immunoblot and RIIβ binding assays yielded similar results (Figure 1, C and E). AKAP 150 is abundant in forebrain regions such as olfactory bulb, caudate-putamen, neocortex, and hippocampus. The hypothalamus and thalamus had considerably lower levels of the anchor protein, whereas brain stem, cerebellum, spinal cord, and pituitary exhibited only faint signals (representative examples are shown in Figure 1C, lane 5 and Figure 1E, lanes 5–7). In all regions, AKAP 150 was present in both the soluble and particulate fractions, but the bulk of the polypeptide was particulate. As noted previously (Bregman *et al.*, 1989, 1991), the occurrence of AKAP 150 in cytosol is probably due to the vigorous mechanical homogenization procedure used to disrupt brain tissue. In cell lines that express AKAP 150 and AKAP 79 ~90% of the anchor proteins are isolated in the particulate fractions after gentle lysis (Hirsch *et al.*, 1992; Glantz, Ndubuka and Rubin, unpublished observations).

The pattern of the regional abundance of RIIβ is similar to that observed for AKAP 150 (Figure 1D). RIIβ content was highest in caudate-putamen and neocortex.

Overview of the Regional Distribution of AKAP 150 and RIIβ

Immunoperoxidase staining of coronal sections of rat brain divulged a distinctive anatomical distribution of anchor protein expression (e.g., Figure 2, A and B).

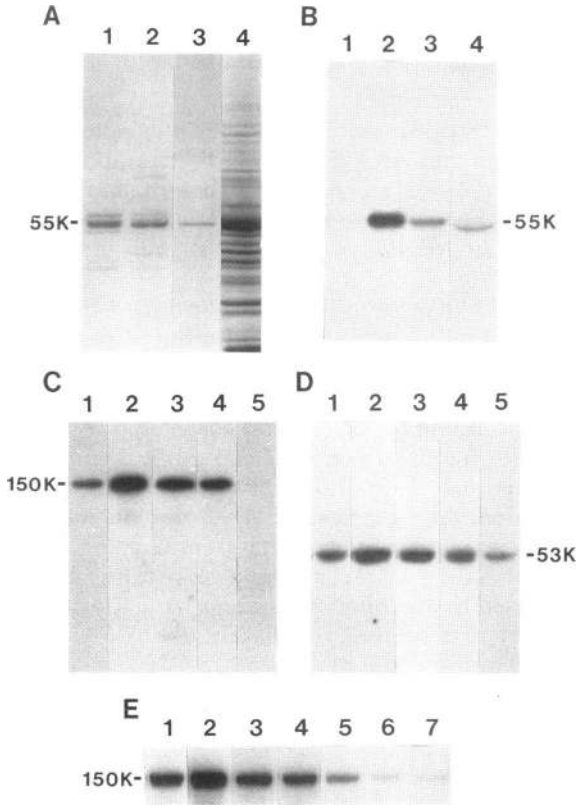

Figure 1. Antibody specificity and the expression of AKAP 150 and RIIβ in dissected brain regions. A Coomassie blue-stained gel (A) and an immunoblot autoradiogram (B) demonstrate the monospecificity of anti-RIIβ IgGs. In A and B, lane 1 contained 1.5 μg of purified bovine heart RIIα; lane 2 received 1.5 μg of purified bovine brain RIIβ; lane 3 contained 0.5 μg of purified recombinant human RIIβ that was expressed in *Escherichia coli;* and lane 4 received 75 μg of rat neocortex homogenate protein. The Western blot was probed with anti-RIIβ IgGs and ^{125}I-protein A and processed as described under MATERIALS AND METHODS. Rat RIIβ has a slightly smaller apparent M_r (~53 000) than bovine and human RIIβ as previously noted by Jahnsen *et al.* (1986). However, the bovine, human, and rat RIIβ subunits each contain 418 amino acids, and the proteins exhibit 97% sequence identity (Luo *et al.*, 1990). Western blots containing polypeptides from the particulate fractions of dissected brain regions were probed with either anti-AKAP 150 antibodies and ^{125}I-protein A (C), anti-RIIβ IgGs and ^{125}I-protein A (D), or ^{32}P-RIIβ (E) as described in MATERIALS AND METHODS. The resulting autoradiograms are presented. The gel lanes contained 75 μg of protein from the particulate fractions of olfactory bulb (lane 1), caudate-putamen (lane 2), neocortex (lane 3), hippocampus (lane 4), hypothalamus (lane 5), cerebellum (lane 6), and brain stem (lane 7). These experiments were repeated 3 times and the results were very similar in each instance. The apparent molecular weights for AKAP 150 and rat RIIβ are 150 000 and 53 000, respectively.

AKAP 150 is abundant in regions of the forebrain including caudate-putamen, nucleus accumbens, olfactory tubercle, hippocampus, neocortex, olfactory bulb, and anterior olfactory nucleus. Substantial levels of AKAP 150 are also evident in the septum and amygdala of the limbic system and in the Purkinje cells of the cerebellum. Only limited amounts of antigen are detected by anti-AKAP 150 IgGs in the thalamus, hypothalamus, midbrain, and hindbrain.

The patterns of distribution and relative abundance of RIIβ are similar to those of AKAP 150 throughout most of the CNS (e.g., Figure 2C).

Localization of AKAP 150 and RIIβ in Rat Brain

The cell-specific enrichment and intracellular targeting of AKAP 150 and RIIβ were analyzed further with higher levels of microscopic resolution.

Basal Ganglia. AKAP 150 and RIIβ are abundant in the basal ganglia and their patterns of localization are strikingly similar (Figure 2, A and C). Intense immunoperoxidase staining revealed high levels of both AKAP 150 and RIIβ in the dendrites of neurons in the caudate-putamen, nucleus accumbens, olfactory tubercle, and the islands of Calleja (Figure 2, A, C, D, and E). Some perikarya (especially in the caudate-putamen) also contain both antigens (Figure 2, D and E). The size, shape, and relative abundance of immunostained neurons in the caudate-putamen and nucleus accumbens indicate that the medium, multipolar spiny neurons are enriched in RIIβ and its anchor protein. High magnification photomicrographs reveal that dendrites from neurons of the ventral pallidum (Figure 2, F and G) also contain significant levels of AKAP 150 and RIIβ. At lower magnification (Figure 2, A and C) this region appears lightly stained because the dendrites are intermingled with numerous unstained axons. Substantial amounts of AKAP 150 and RIIβ are also present in the dendrites of neurons in several amygdaloid nuclei.

Olfactory Bulb and Anterior Olfactory Nucleus. High levels of AKAP 150 and RIIβ immunoreactivity are apparent in two layers that contain the dendrites of the granule and periglomerular cells of the olfactory bulb (Figure 3, A–E, G, and H). Dendrites of neurons within the anterior olfactory nucleus also contain considerable amounts of both RIIβ and AKAP 150 (Figure 3, F and I). In contrast, axons of the olfactory nerve and the lateral olfactory tract are not stained.

Cerebral Cortex. Strong signals for AKAP 150 are observed throughout the neocortex (Figure 4). Neurons in each of the cortical layers contain AKAP 150, but the pyramidal neurons of layers III and V stain most intensely (Figure 4, A and B). AKAP 150 is considerably more abundant in the apical dendrites of pyramidal neurons than in their basal dendrites. The weaker staining of the fine dendritic branches in the outer molecular layer shows that AKAP 150 is largely confined to primary dendrites (Figure 4, A and B). Most neuronal perikarya are only lightly stained. However, the anchor protein is clearly evident in the perikarya of the largest pyramidal neurons (Figure 4B).

RIIβ is observed in neurons throughout the neocortex. It accumulates primarily in the dendritic compartment of neocortical neurons where AKAP 150 is also abun-

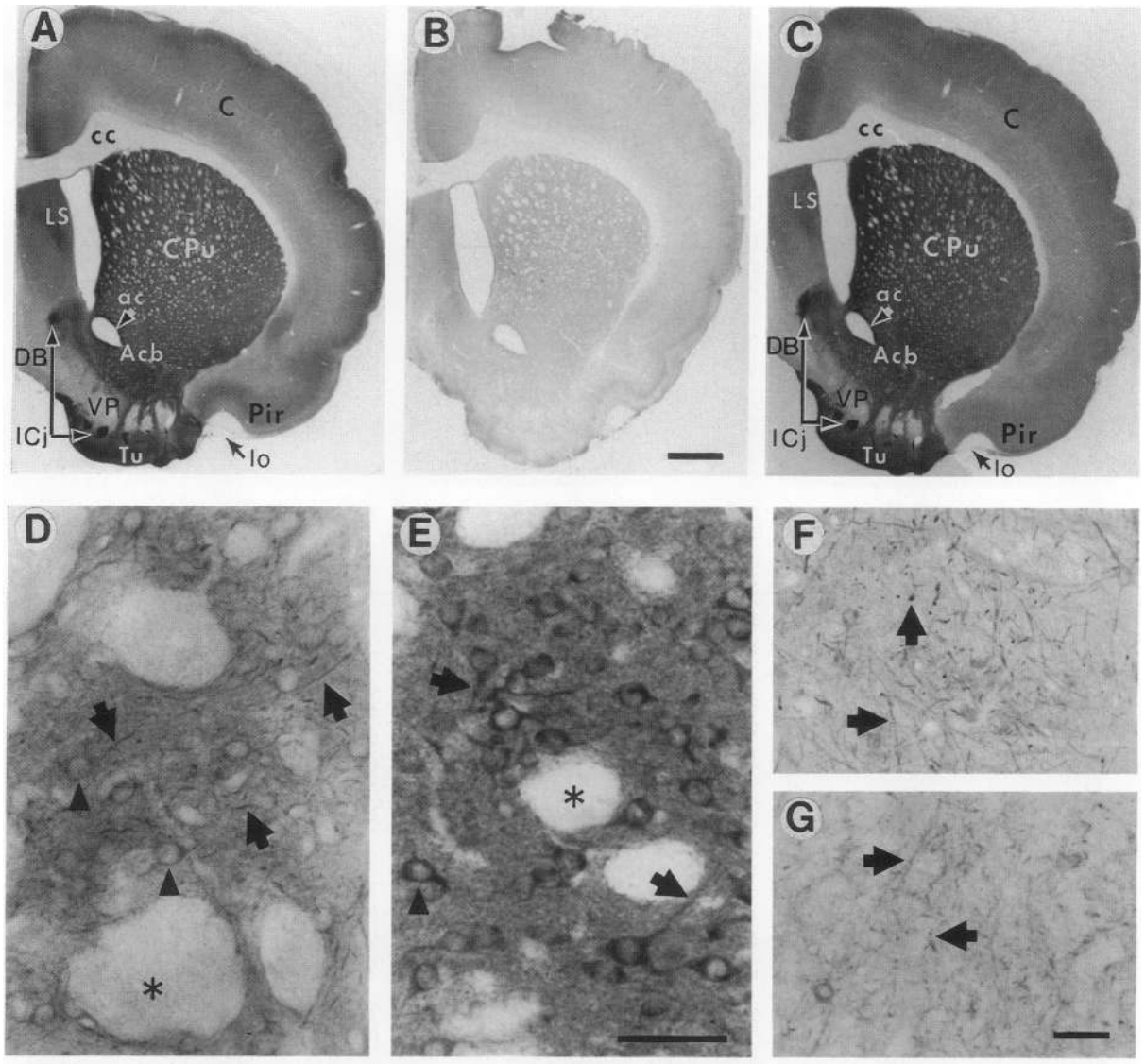

Figure 2. Distribution of AKAP 150 and RIIβ in the basal ganglia. Immunoperoxidase staining of sections was performed as described in MATERIALS AND METHODS with preimmune serum (B) or primary antibodies directed against either AKAP 150 (A, D, and F) or RIIβ (C, E, and G). The immunostaining of coronal sections of the forebrain (A–C) revealed high levels of AKAP 150 and RIIβ in basal ganglia regions including the caudate-putamen (CPu), nucleus accumbens (Acb), olfactory tubercle (Tu), and the islands of Calleja (ICj). AKAP 150 and RIIβ were also abundant in the neocortex (C), piriform cortex (Pir), and lateral septum (LS), whereas the ventral pallidum (VP) and diagonal band (DB) contained only moderate levels of the 2 antigens. Fiber tracts such as the corpus callosum (cc), anterior commissure (ac), and the lateral olfactory tract (lo) were deficient in both proteins. (D and E) High magnification photomicrographs of the caudal caudate-putamen. AKAP 150 and RIIβ immunoreactivity was evident in neuronal perikarya (◀), primary dendrites (←), and in the processes of the surrounding neuropil. Perikaryal staining was more prominent in the caudal (vs. rostral) caudate-putamen. Nuclei were unstained. Asterisks indicate internal capsule axon fibers, which showed little or no positive staining. (F and G) High magnification photomicrographs of the ventral pallidum. Positive staining was present in dendrites (←). Bar, 1 mm (A–C); 50 μm (D and E); 50 μm (F and G).

dant (Figure 4, A and C). RIIβ and AKAP 150 are also detected in perikarya (Figure 4, A–C). However, considerable RIIβ immunoreactivity is apparent in the terminal dendritic branches in the outer molecular layer (Figure 4C) where relatively weak staining is observed with antibodies directed against AKAP 150 (Figure 4A).

Overall, the distribution of RIIβ immunoreactivity in the neocortex (and other forebrain regions) is consistently more diffuse than that of AKAP 150. This is due to the differential accumulation of RIIβ in the neuropil, which corresponds to the finer dendritic branches and possibly, axon terminals.

Neurons in piriform cortex (Figure 2, A and C), entorhinal cortex, and hippocampus (see below) also contain both AKAP 150 and RIIβ in their dendrites. No significant staining is observed for either AKAP 150 or

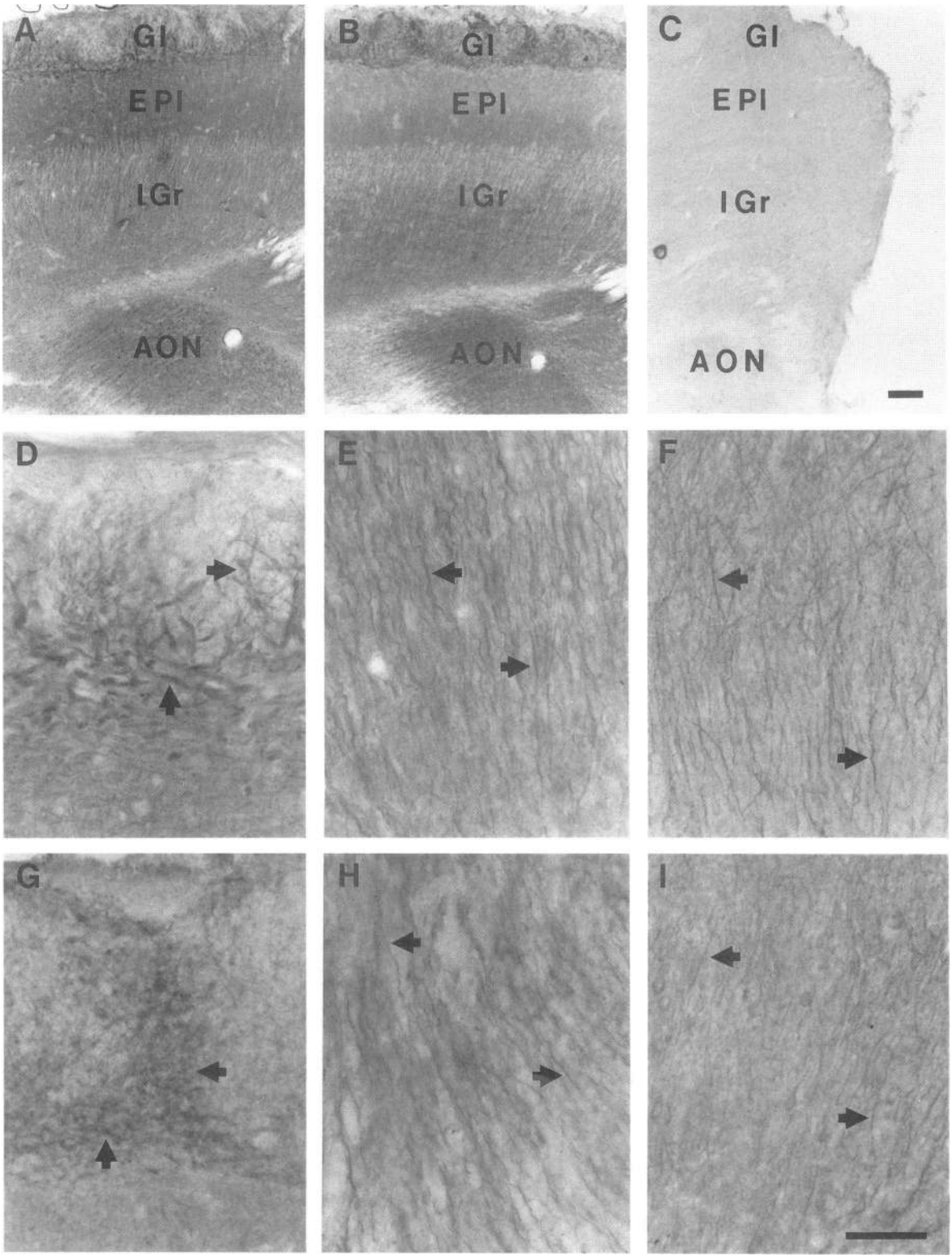

Figure 3. Distribution of AKAP 150 and RIIβ in the olfactory bulb and anterior olfactory nucleus. Vibratome sections of the olfactory bulb and anterior olfactory nucleus were subjected to immunoperoxidase staining with either anti-AKAP 150 (A, D, E, and F), anti-RIIβ (B, G, H, and I) or preimmune (C) IgGs as the primary antibody. The peroxidase reaction product corresponding to AKAP 150 was abundant in the dendrites of the glomerular (Gl) and internal granular (IGr) layers of the olfactory bulb and in the anterior olfactory nucleus (AON) dendrites

RIIβ in individual cortical axons or distally in the corpus callosum (Figure 2, A and C).

Hippocampus. Primary apical dendrites of pyramidal neurons in the CA1, CA2, and CA3 regions exhibit high levels of AKAP 150 immunoreactivity (Figure 5, A and C). AKAP 150 is also present in the basal dendrites of pyramidal neurons, dendrites of granule cells (dentate gyrus) and dendrites originating from the hilus cells (Figure 5, A, C–E). Only a minimal level of anchor protein immunoreactivity is detected in neuronal perikarya in these regions.

RIIβ is colocalized with AKAP 150 in the apical and basal dendrites of pyramidal neurons in the CA1-CA3 regions of the hippocampus, as well as in granule cell (dentate gyrus) and hilus neuron dendrites (Figure 5, B, F–H). However, some features of the staining patterns for RIIβ and AKAP 150 differ. For example, RIIβ is more evident in the perikarya and fine peripheral branches of dendrites of pyramidal neurons (Figure 5, F and G). It appears that RIIβ is targeted to dendrites in all of the pyramidal cells in CA1 (Figure 5F). In contrast, only a subset of pyramidal neurons express substantial levels of RIIβ in CA2 and CA3 (Figure 5G). Furthermore, the stratum lucidum of CA3 contains RIIβ in an AKAP 150-deficient region that corresponds to the terminals of the mossy fiber from the dentate gyrus (Figure 5, B and G). The granule cell axons that form the mossy fiber in the hilus region are not visualized with the anti-RIIβ IgGs (Figure 5H).

Cerebellum. AKAP 150 is abundant only in the primary dendrites of Purkinje cells of the cerebellar cortex (Figure 6A). The small peripheral branches of these dendrites are not visualized via the immunoperoxidase procedure. Although some staining for AKAP 150 is apparent in Purkinje cell bodies, the signal only slightly exceeds that observed with preimmune serum (Figure 6, A and C). Low levels of RIIβ are also present in the Purkinje cell dendrites and perikarya (Figure 6B).

Thalamus, Hypothalamus, Midbrain, and Hindbrain. AKAP 150 and RIIβ immunoreactivities are quite low in neurons throughout the thalamus, hypothalamus, midbrain, and hindbrain (Figure 7, A–D). Nevertheless, the targeting of AKAP 150 and RIIβ to dendrites and some perikarya is apparent in neurons of the medial habenula, substantia nigra, superficial layer of the superior colliculus, the supraoptic, medial tuberal and interpeduncular nuclei, and the ventromedial hypothalamus (e.g., see Figure 7, A–D). The area encompassing the third ventricle and the sylvian aqueduct is atypical. Neurons in the periventricular, arcuate and dorsomedial hypothalamic nuclei, the median eminence, and the ventral periaqueductal region are deficient in AKAP 150 but stain with anti-RIIβ-selective IgGs (Figure 7, E–G).

AKAP 150 Is Targeted to Microtubules in the Dendritic Cytoskeleton

The intracellular localization of AKAP 150 in hippocampal pyramidal neurons and Purkinje cells was analyzed at a higher level of resolution by combining immunoperoxidase staining with electron microscopy. In both types of neurons the peroxidase reaction product is selectively associated with dendritic microtubules (Figure 8, B–D). No staining of microtubules or other structures is observed when anti-AKAP 150 serum is replaced with preimmune serum (Figure 8A).

DISCUSSION

AKAPs have been implicated in the targeting of cAMP signaling in brain on the basis of their high affinity interactions with RIIβ in vitro and their tissue-restricted expression and relative abundance in the CNS (Sarkar *et al.*, 1984; Leiser *et al.*, 1986; Bregman *et al.*, 1989, 1991; Luo *et al.*, 1990). However, evidence documenting the coaccumulation of an AKAP and RIIβ in specific types of neurons and at a distinct intraneuronal site was lacking.

We detected AKAP 150 in many of the classes of neurons that constitute the rat CNS (Figures 2–7). However, the level of AKAP 150 immunoreactivity varies markedly as a function of brain region and neuronal cell type (e.g., Figures 1–3 and 7). AKAP 150 is abundant in the pyramidal neurons of the cerebral cortex (Figures 4 and 5), the medium spiny neurons of the caudate-putamen and nucleus accumbens (Figure 2), the granule cells of the olfactory bulb (Figure 3) and dentate gyrus (Figure 5), and cerebellar Purkinje cells (Figure 6). A very high proportion of neuronal AKAP 150 is sequestered in a morphologically and functionally specialized subcellular region—microtubules of the proximal dendritic cytoskeleton (Figure 8).

The content of AKAP 150 in the majority of neurons of the thalamus, hypothalamus, midbrain, and hindbrain (excluding Purkinje cells) can be characterized qualitatively as modest or extremely low (see Figure 7). The anchor protein is not detected in non-neuronal cells, although the presence of a low level of AKAP 150 in such cells is not excluded by the methodology used.

(A). The pattern of RIIβ immunoreactivity generally matched the distribution of AKAP 150 immunoreactivity (B). The more diffuse staining of AKAP 150 and RIIβ in the external plexiform layer (EPl) is not associated with the mitral dendrites that are prominent in this region. (D and G) High magnification photomicrographs demonstrate the accumulation of AKAP 150 and RIIβ in periglomerular dendrites (←) of the glomerular layer of the olfactory bulb. (E and H) Granule cell dendrites (←) of the internal granular layer contain significant amounts of both AKAP 150 and RIIβ. (F and I) AKAP 150 and RIIβ are readily detected in individual dendrites of the anterior olfactory nucleus. Bar, 100 μm (A–C); 50 μm (D–I).

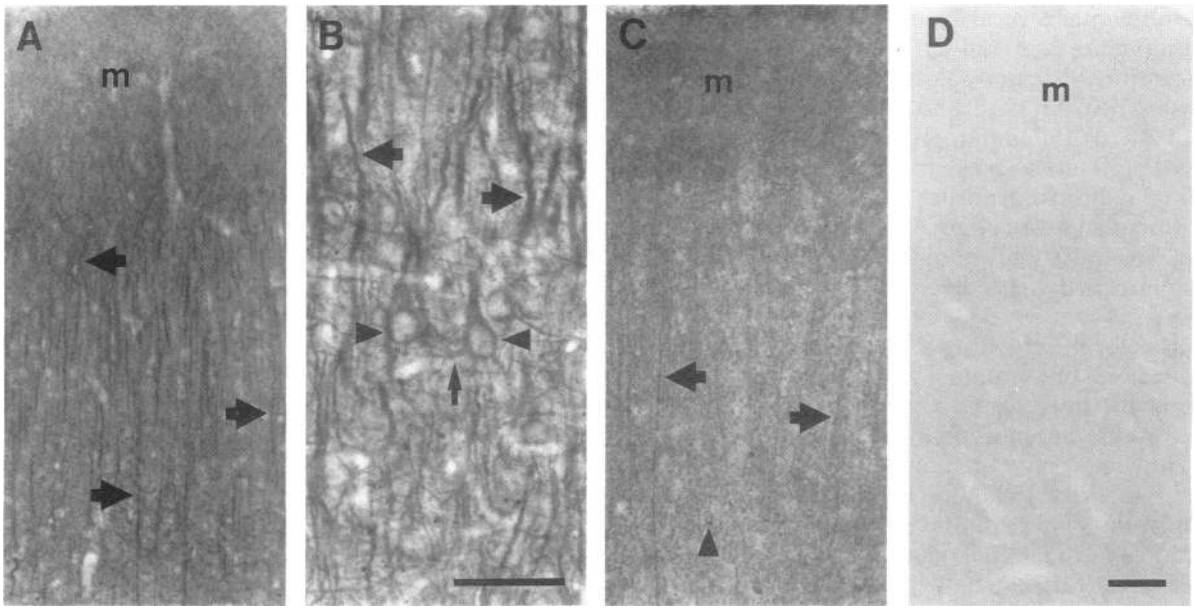

Figure 4. Distribution of AKAP 150 and RIIβ in the neocortex. Immunoperoxidase staining of the neocortex was performed with anti-AKAP 150 (A and B), anti-RIIβ (C), and preimmune (D) IgGs. AKAP 150 accumulated in neurons in all of the neocortical layers. (A) Outer layers (I-III). The highest levels of AKAP 150 were observed in primary apical dendrites (large arrows) of pyramidal neurons (A and B). Intermediate levels of AKAP 150 immunoreactivity were seen in perikarya (arrowheads) and basal dendrites (small arrow) of the pyramidal cells (B). The outermost, molecular layer (m) of the neocortex, which contains small, peripheral dendritic branches, exhibited a low level of AKAP 150 immunoreactivity (A). RIIβ accumulated primarily in the dendrites (large arrows) and also in perikarya (arrowhead) of neurons in each neocortical layer (C). The molecular layer and cortical neuropil stained more intensely for RIIβ than AKAP 150, thereby producing a more diffuse pattern of RIIβ localization. Bar, 50 μm (A, C, and D); 50 μm (B).

Brain regions, specific neurons, and intraneuronal sites that contain substantial levels of RIIβ (and PKAIIβ) are generally coincident with areas abundant in AKAP 150 throughout most of the CNS (Figures 1–7).

In previous ultrastructural studies we (Ludvig *et al.*, 1990) and others (Trinczek and Schwoch, 1990) demonstrated that both RIIβ and catalytic subunits of cAMP-dependent protein kinase are concentrated in dendrites in close apposition with microtubules. Immunoperoxidase staining coupled with electron microscopy now reveals that AKAP 150 is selectively associated with microtubules in primary dendrites (Figure 8).

Our observations on the colocalization of RIIβ and AKAP 150 in subsets of neurons provide strong support for a working model in which the anchor protein tethers PKAIIβ near dendritic microtubules. A high concentration of PKAIIβ would be established in proximity with both neurotransmitter-activated adenylate cyclase in the synaptic plasma membrane and substrate proteins in the cytoskeleton. Such an arrangement would ensure the efficient reception, amplification, and propagation of signals carried by the second messenger cAMP. The ability of PKAIIβ to phosphorylate and functionally modify microtubule-associated protein 2 (MAP2) and neurotransmitter receptors provides mechanisms for 1) transmitting signals via cytoskeletal interconnections and 2) desensitizing synapses to subsequent pulses of neurotransmitter, respectively (reviewed in Hemmings *et al.*, 1987, 1989; Gelfand and Bershadsky, 1991; Collins *et al.*, 1992).

MAP2 is a brain-enriched RII binding protein that is not homologous with the AKAPs. It binds both RII isoforms at a site near its N terminus, but its affinity for RIIα is 7-fold higher (Obar *et al.*, 1989; Rubino *et al.*, 1989). Moreover, RIIα co-purifies with MAP2 under certain conditions (Theurkauf and Vallee, 1982). MAP2 is abundant in brain ($\geq$ concentration of RII subunits) and apparently plays important structural roles in the assembly and stabilization of neuronal microtubules (Gelfand and Bershadsky, 1991). MAP2 accumulates uniformly within the dendrites of virtually all neurons (Bernhardt and Matus, 1984; De Camilli *et al.*, 1984).

The concentration of the AKAP homologs in brain is much lower ($\leq$ concentration of RII) than that of MAP2, and these proteins preferentially complex and co-purify with RIIβ (Sarkar *et al.*, 1984). The lower abundance, differential expression and more selective intracellular targeting of AKAP 150 in various neurons, suggest that this anchor protein subserves a different (regulatory ?) function than MAP2. Nonetheless, both AKAP 150 and MAP2 coexist in the primary dendrites of many neurons and both bind RIIβ and RIIα. Ultimately, it will be necessary to determine 1) whether the two anchor proteins contain segregated or interacting pools of RII tethering

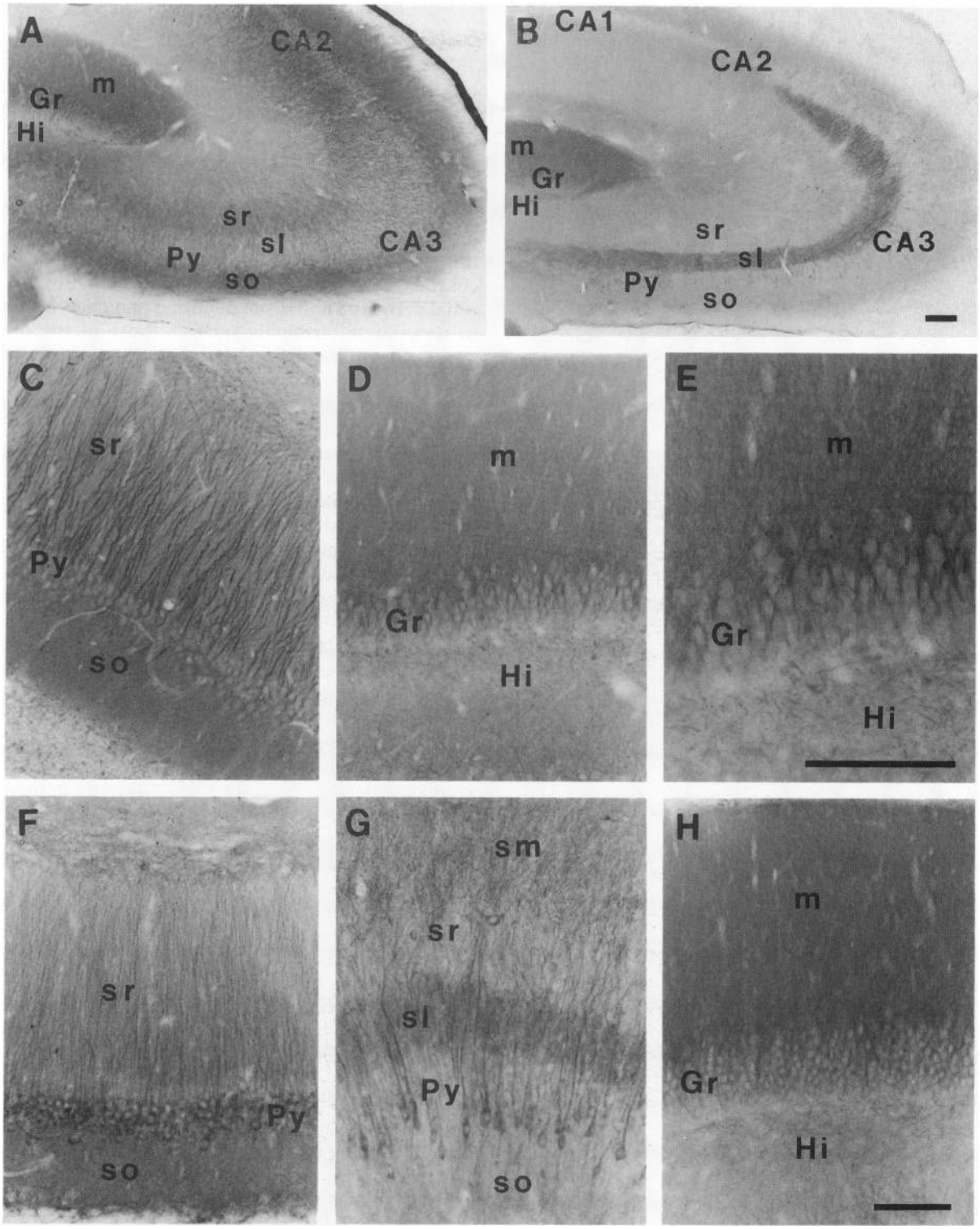

Figure 5. Distribution of AKAP 150 and RIIβ in the hippocampus. Vibratome sections of the hippocampus were subjected to immunoperoxidase staining for AKAP 150 (A, C, D, and E) and RIIβ (B, F, G, and H). In the hippocampus, the dendrites of pyramidal neurons (Py) of Ammon's horn (CA1, CA2, and CA3 areas) and the granule neurons (Gr) of the dentate gyrus contained AKAP 150 (A). At higher magnification AKAP 150 immunoreactivity was clearly evident and abundant in the apical pyramidal cell dendrites of the stratum radiatum (sr), the smaller basal pyramidal cell dendrites of the stratum oriens (so), the granule cell dendrites of the dentate gyrus molecular layer (m) and the dendrites of the hilus neurons (C-E). Perikaryal staining was negligible. The RIIβ antigen was most prominent in the dentate gyrus and the stratum lucidum (sl) of CA3 (B). Higher magnification photomicrographs of more caudal hippocampal regions show the accumulation of RIIβ in pyramidal cell dendrites in CA1 (F), a subpopulation of the pyramidal cell dendrites of CA2 and CA3 (G), dentate gyrus granule cell dendrites and the hilus neuron dendrites (H). The presence of RIIβ in the pyramidal perikarya, the finer pyramidal dendritic branches of the stratum moleculare (sm) and in a few other interneurons is also evident (G). RIIβ in the stratum lucidum of CA3 appeared to be associated with the mossy fiber axon terminals (B and G). Bar, 100 μm (A and B); 100 μm (C, D, F, G, and H); 100 μm (E).

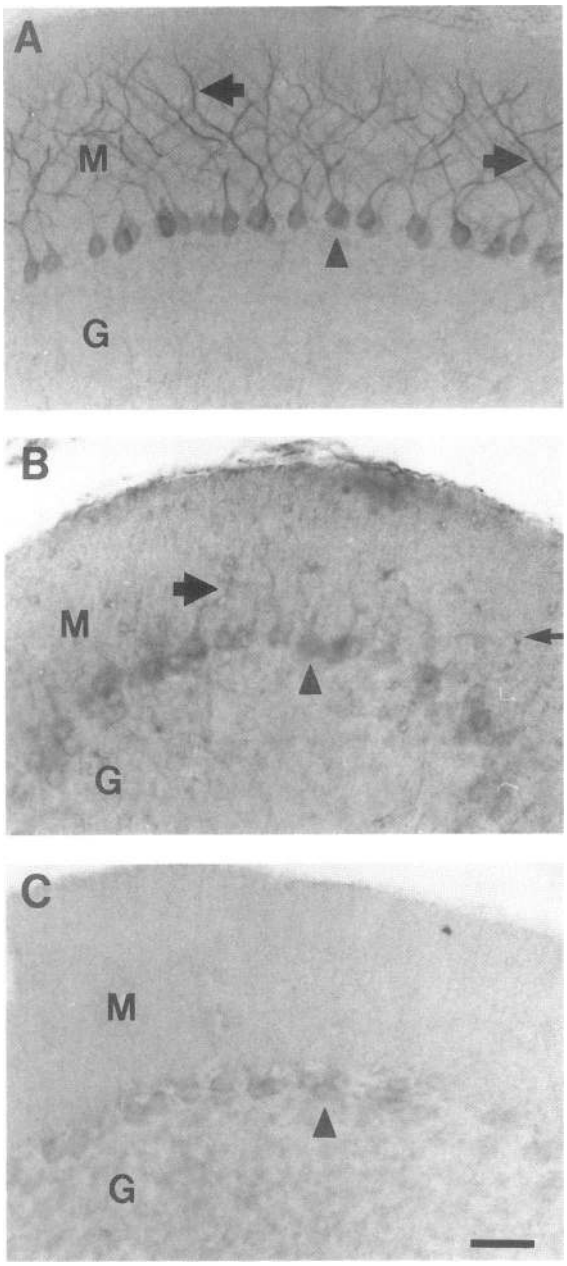

Figure 6. Distribution of AKAP 150 and RIIβ in the cerebellar cortex. Sections of the cerebellar cortex were subjected to immunoperoxidase staining with anti-AKAP 150 (A), anti-RIIβ (B), or preimmune (C) IgGs as the primary antibody. The locations of the molecular (M) and granular (G) layers are indicated. AKAP 150 immunoreactivity was abundant only in Purkinje neurons. The anchor protein was localized to the dendrites (large arrows); the finest peripheral branches were not well stained. Purkinje cell perikaryal staining (arrowheads) was only slightly more intense than that observed with the preimmune serum. A low level of RIIβ immunoreactivity was observed in the Purkinje neurons (perikarya and dendrites) and in a few other neurons such as the stellate neurons of the molecular layer (small arrow). Bar, 50 μm.

sites and 2) what mechanisms govern the distribution of RIIβ and RIIα between AKAP 150 and MAP2.

Purkinje cell dendrites are heavily stained with anti-AKAP 150 antibodies, whereas only a modest level of RIIβ immunoreactivity is evident (Figure 6). This suggests that, in addition to anchoring PKAIIβ, AKAP 150 might also play a role in modulating the properties of the cytoskeleton that is independent of the kinase. In contrast, RIIβ is more abundant than AKAP 150 in neuronal perikarya and distal dendritic branches. Moreover, in some axon terminals such as those of the dentate gyrus mossy fiber, RIIβ is detected although AKAP 150 immunostaining is absent (Figure 5). These results are in accord with established roles for PKA in regulating cytoplasmic metabolic enzymes (Edelman *et al.*, 1987), the observation that ~30% of PKAIIβ is isolated in brain cytosol (Stein *et al.*, 1987), the ability of RIIβ to bind with the ubiquitous MAP2 and the presence of PKAIIβ in presynaptic structures. Clearly, only a subset of neuronal PKAIIβ molecules is linked to AKAP 150 and the cAMP-stimulated kinase performs AKAP 150-independent functions in several intraneuronal compartments.

Previously, Cadd and McKnight (1989) characterized the expression of RIIβ and RIIα mRNAs in various regions of murine brain by in situ hybridization. RIIβ mRNA was much more prominent than RIIα mRNA, and the patterns of expression of the two transcripts were distinct. Especially high signals for RIIβ mRNA were observed in cerebral cortex, caudate-putamen and dentate gyrus, whereas RIIα mRNA was abundant only in the medial habenula. Our results indicate that brain regions enriched in RIIβ mRNA contain elevated amounts of the cognate polypeptide.

RIIα, the principal RII isoform outside of the CNS, also accumulates in brain, albeit at low levels (Stein *et al.*, 1987). De Camilli and co-workers (1986) used anti-RIIα antibodies to demonstrate the presence of this isoform in the perikarya and dendrites of neurons in the neocortex and hippocampus (pyramidal cells), medial habenula, cerebellum (Golgi cells), hypothalamus, and globus pallidus. Electron microscopy revealed that RIIα was associated with microtubule organizing centers, microtubules, and the Golgi apparatus (De Camilli *et al.*, 1986). RIIα colocalized with MAP2, but the possibility that some RIIα subunits occupy binding sites on AKAP 150 has not been excluded. The lower content and restricted distribution of RIIα suggest that PKAIIα plays a more limited role than PKAIIβ in signal transduction in the CNS.

A clue regarding AKAP function can be deduced by correlating experimental results reported here with information obtained through the elegant studies of Greengard and co-workers (reviewed in Hemmings *et al.*, 1987, 1989) on the medium spiny neurons of the caudate-putamen. Occupation of the dopaminergic D1 receptors on the medium spiny neurons with agonists

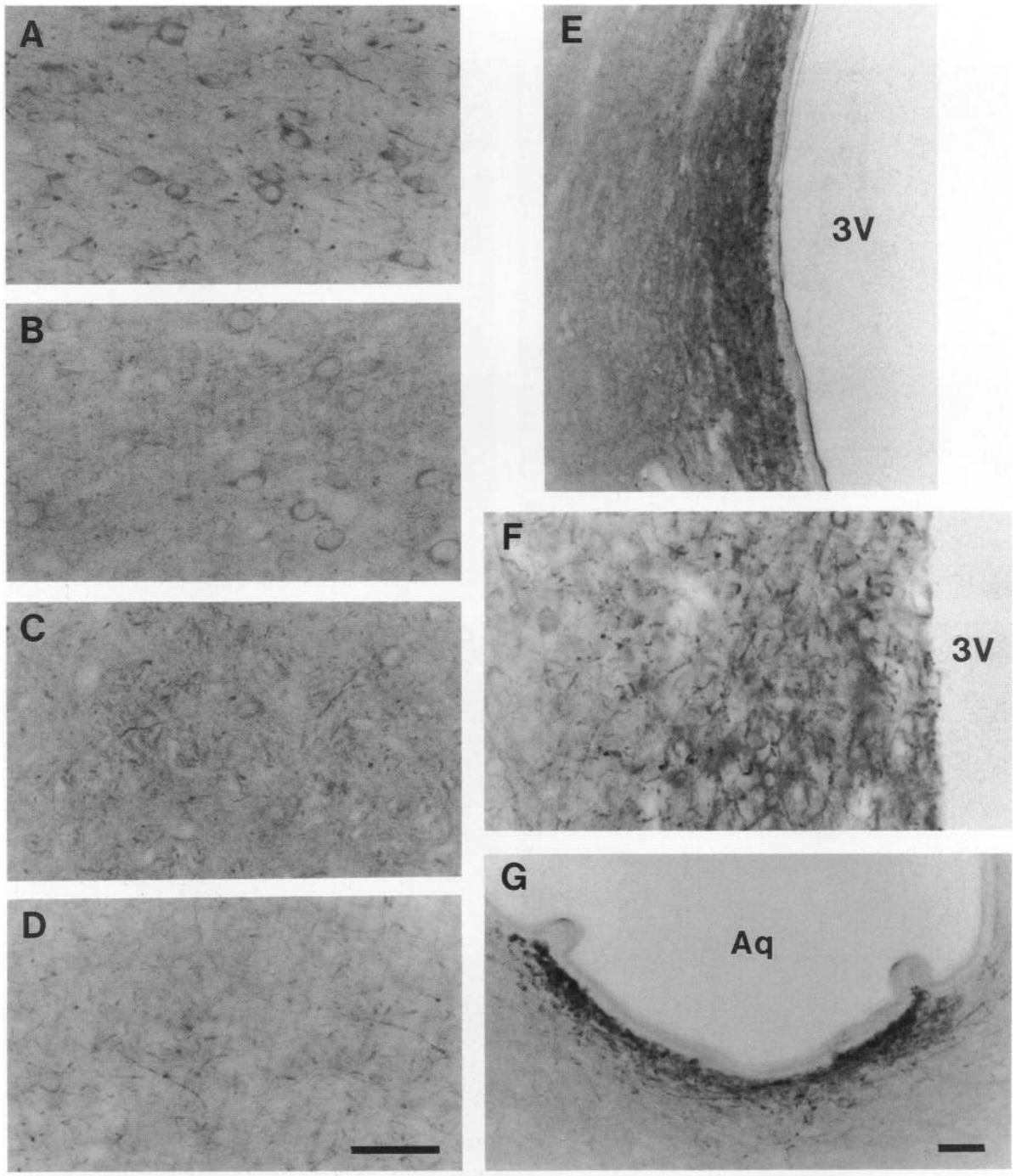

Figure 7. Distribution of AKAP 150 and RIIβ in regions of the thalamus, hypothalamus, midbrain, and hindbrain. Sections were stained for AKAP 150 (A and C) and RIIβ (B, D, E, F, and G) with the immunoperoxidase technique. Weak to (occasional) moderate signals for AKAP 150 and RIIβ were observed in the dendrites and some perikarya in most regions. The ventral thalamus (A and B) and the substantia nigra reticulata (C and D) are shown as examples. Regions of the hypothalamus and midbrain that surround the third ventricle (3V) and sylvian aqueduct (Aq) exhibited staining for RIIβ (E–G) in the absence of AKAP 150 immunoreactivity (not shown). The periventricular hypothalamic nucleus (E), the arcuate hypothalamic nucleus (F) and the periaqueductal region (G) are shown. Ependymal cells lining the sylvian aqueduct and the third ventricle were not stained. Bar, 50 μm (A, B, C, D, and F); 50 μm (E and G).

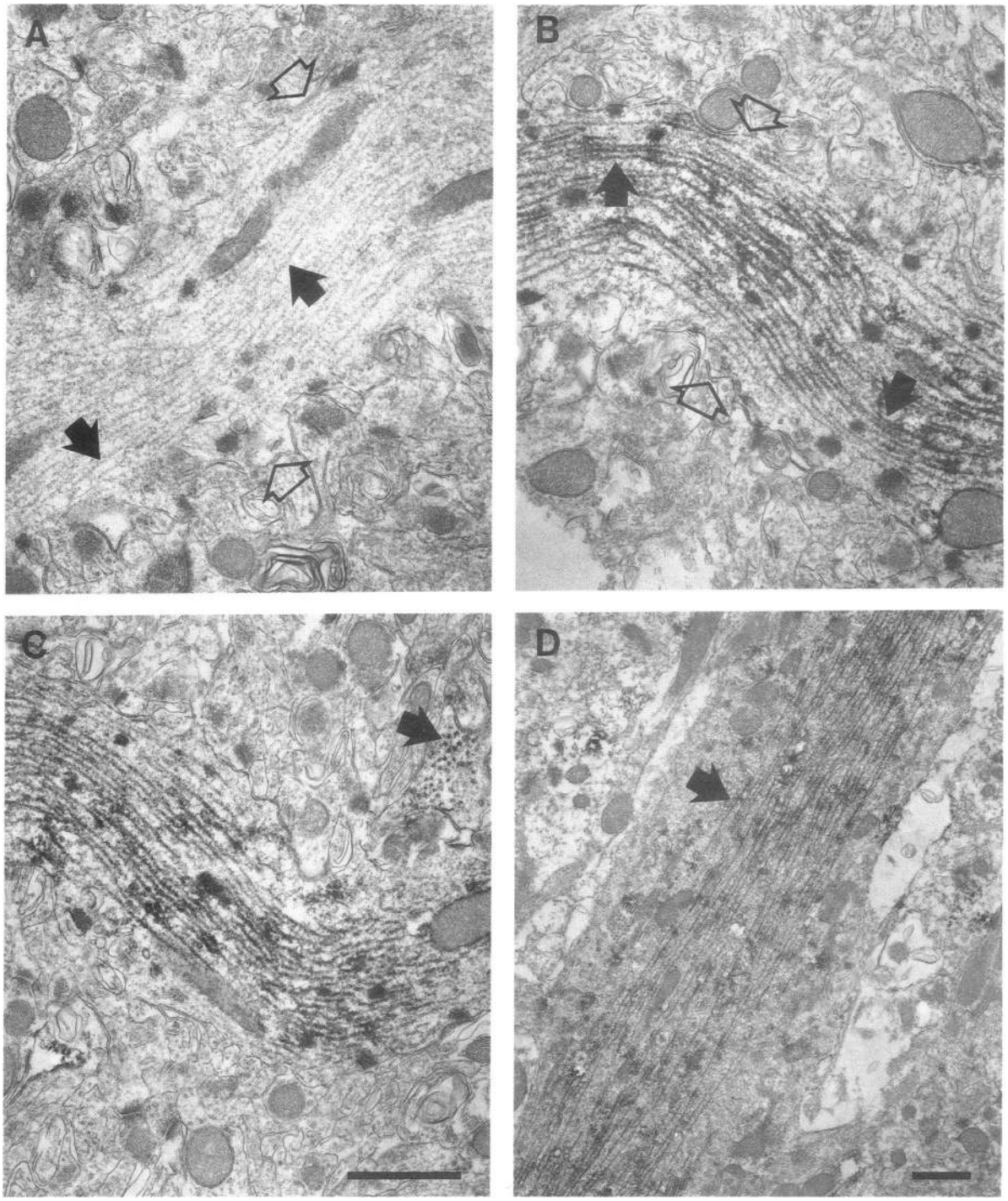

Figure 8. Intracellular localization of AKAP 150 in cerebellar Purkinje cells and hippocampal pyramidal neurons. Sections containing dendrites of hippocampal pyramidal neurons (A–C) or Purkinje cells (D) were stained by the immunoperoxidase procedure with preimmune (A) or anti-AKAP 150 (B–D) serum. The specimens were then processed for electron microscopy as described under MATERIALS AND METHODS. The peroxidase reaction product is visualized as dark granular material. AKAP 150 is closely associated with the microtubules (←) in the dendrites of both the pyramidal (B and C) and Purkinje (D) neurons. Open arrows indicate plasma membrane-dendrite boundaries. (C) Colocalization of AKAP 150 with microtubules is also observed in a dendrite cross-section where the reaction product is deposited around small circular structures (←) that correspond to individual microtubules. Bar, 1 μm (A–C); 1 μm (D).

results in the activation of adenylate cyclase and the subsequent phosphorylation of DARP-32 (dopamine and cAMP-regulated phosphoprotein of 32 kDa). The cAMP-mediated phosphorylation of a Thr residue in inactive DARP-32 converts the target protein into a potent inhibitor of protein phosphatase 1 (Hemmings *et al.*, 1987, 1989). Although a substantial body of experimentation has defined the nature and locations of the neurotransmitter, the D1 receptor, and DARP-32, little is known about which PKA isoform is involved and where it is localized in the neurons. The accumulation of AKAP 150 and RIIβ at or near sites of signal reception in the medium spiny neurons of the caudate putamen (Figure 2) suggests the speculations that 1) AKAP 150-PKAIIβ complexes in the dendritic cytoskeleton constitute intracellular target sites for dopamine-initiated, cAMP signaling and 2) the PKAIIβ isoform might be the major mediator of this signal transduction pathway in these neurons.

Receptor/transmitter mapping studies (e.g., Rainbow *et al.*, 1984; Dumuis *et al.*, 1988; Alexander and Reddington, 1989; McLean *et al.*, 1989; Fremeau *et al.*, 1991) suggest that many neurons that contain adenylate cyclase-linked receptors for dopamine, norepinephrine, serotonin, and adenosine are also enriched in both AKAP 150 and RIIβ (see Figures 2–6). As data accumulate on these signaling systems, it will become feasible to determine whether AKAP 150-RIIβ complexes are characteristic components of such pathways.

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