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Calcium-binding proteins are concentrated in the CA2 field of the monkey hippocampus: a possible key to this region's resistance to epileptic damage

C. Leranth[1] and C.E. Ribak[2]

[1] Department of Obstetrics and Gynecology and Section of Neuroanatomy, Yale University, School of Medicine, P.O. Box 3333, 333 Cedar Street, New Haven, CT 06510, USA
[2] Department of Anatomy and Neurobiology, University of California, College of Medicine, Irvine, CA 92717, USA

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Summary. Previous immunocytochemical studies have shown a heterogeneous distribution of parvalbumin (PA) and calbindin (CB) in the rat hippocampal formation. The results of the present study showed a heterogeneous distribution of PA and CB in primate Ammon's horn. The density and intensity of immunoreactivity for both of these calcium-binding proteins was greatest in CA2 as compared to CA1 and CA3. CB-immunoreactivity was localized to the cell bodies, dendrites, and axon initial segments of pyramidal cells whereas PA-immunostaining was found in the axon terminals, dendrites and cell bodies of interneurons that have features similar to GABAergic inhibitory neurons. Based on previous studies that have shown a protective role of calcium-binding proteins in neurons exposed to hyperstimulation, these results suggest that the resistance of CA2 pyramidal cells in temporal lobe epilepsy is due to the high concentration of CB and PA in this region of Ammon's horn.

Key words: Calbindin – Parvalbumin – Pyramidal cells – GABAergic neurons – Epilepsy – Monkey

Introduction

In patients suffering from temporal lobe epilepsy a selective pattern of hippocampal cell loss, referred to as "hippocampal sclerosis", is a well documented observation (see review in Babb et al. 1984; Bruton 1988; Dam 1982; Sloviter 1983). The major loss of neurons is observed in the CA1 and CA3 subfields of the Ammon's horn and in the hilus of the dentate gyrus. The granule cells, and according to a recent statistical analysis (Kim et al. 1990), more than 50% of the CA2, so called "resistant zone" pyramidal cells, are spared. A similar pattern of hippocampal cell loss was reported for rats by Sloviter (1983) after sustained electrical stimulation of the entorhinal cortex. It was suggested that the seizure-associated brain damage in rats is caused by excessive presynaptic release of excitatory transmitters that induces intracellular postsynaptic changes leading to cell death.

In the past decade, the importance of calcium-binding proteins has been realized by the neuroscience community (see review by Celio 1990). The majority of these studies focused on the localization and function of these proteins in the rat. For example, Sloviter (1989) has plotted the immunoreactivity for calbindin (CB) and parvalbumin (PA) in the rat and speculated that these proteins have a protective function because neurons with intense immunoreactivity for calcium-binding proteins are not affected by intense stimulation used in his experimental paradigm. Furthermore, Scharfman and Schwartzkroin (1989) demonstrated that hyperstimulated mossy cells in the hilus of the dentate gyrus became resistant to dying after they were intracellularly injected with a calcium chelator. This view of the protective role of calcium-binding proteins has been further supported in that somatostatin- and neuropeptide Y-containing hippocampal neurons die in experimentally induced (Sloviter 1987) and temporal lobe epilepsy (deLanerolle et al. 1989), and colocalization experiments demonstrate that less than 6% of the somatostatin neurons of the rat fascia dentata contain PA (Nitsch et al. 1990a).

Studies in the primate hippocampus regarding the localization of calcium-binding proteins are sparse. Only the report of Braak et al. (1989) has indicated that GABAergic local circuit neurons in the human hippocampus contain PA. Therefore, the present analysis addresses the distribution of two calcium-binding proteins, PA and CB, in the primate hippocampus. We will demonstrate that the pattern of immunoreactivity for both of these protective proteins displays a dense concentration in the CA2 field of the Ammon's horn, whereas the other subfields, CA1 and CA3 are much less densely immunostained.

Offprint requests to: C. Leranth, Department of Obstetrics and Gynecology (address see above)

Material and methods

Animals

Three adult female (4.5–5 kg B.W) and two adult male (5.5–6 kg B.W) African green monkeys *(Cercopithecus aethiops)* kept on standard monkey pellets and tap water ad libitum, were used in this experiment. The animals were housed in large social cages in the St. Kitts Biomedical Research Foundation. Before the start of the experiment monkeys were placed in individual cages. The St. Kitts facility is in full compliance with all applicable U.S. regulations and the U.S.P.H.S. Guide for Use of Animals, and has provided an assurance of compliance no A3005 to O.P.R.R.R. The facility traps or breeds all of its own animals and has a complete 48 h veterinary service.

Tissue processing

After i.m. Ketamine anesthesia, the animals were sacrificed with sodium pentobarbital administered intravenously and transcardially perfused with 1L of heparinized saline followed by 2–2.5 L of fixative containing 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M (pH 7.4) phosphate buffer (PB). The brains were rapidly removed and postfixed for 2 h at 4° C in the same fixative, except that it lacked glutaraldehyde. After postfixation, the material was further stored at 4° C in PB containing 0.1% NaN_3 and transported to the research laboratory at Yale University.

Immunostaining

After dissecting the hippocampal formation, vibratome sections (60 μm) were cut perpendicular to the longitudinal axis of the primate hippocampal formation, and sections were obtained from two representative areas, the body and head. The sections were washed several times in PB, transferred into a vial containing 0.5 ml of 10% sucrose in PB, rapidly frozen by immersing the vial into liquid nitrogen and then thawed to room temperature. Following several more PB washes, sections were incubated for 20 minutes in sodium borohydride (1% in PB) to remove unbound aldehydes (Kosaka et al. 1986). Consecutive sections were immunostained either for parvalbumin (PA) or calbindin (CB) by incubating the sections for 48 h at 4° C in a monoclonal antibody directed against PA (Celio et al. 1988) or in a rabbit polyclonal antiserum raised against CB (Baimbridge and Miller 1982), respectively. The PA antibody was diluted (1:20,000) in PB containing 1% normal horse serum and 0.1% sodium azide. The CB antiserum was diluted (1:5,000) in PB containing 1% normal goat serum and 0.1% sodium azide. After extensive washing in PB the sections were further processed using the ABC technique of Hsu et al. (1981) and the Vectastain Elite Kit (Vector Laboratories, Burlingame, CA). The tissue bound peroxidase was visualized with a diaminobenzidine (DAB) reaction (14.5 mg DAB, 165 μl 0.3% H_2O_2 in 25 ml PB for 5–10 minutes). After several rinses in PB the sections were osmicated (1% OsO_4 in PB), dehydrated in graded ethanol (the 70% ethanol contained 1% uranyl acetate) and embedded for electron microscopic sectioning. Ultrathin sections were cut on Reichert-Jung Ultracut-E and Sorvall MT–5000 microtomes, collected on Formvar coated single slot grids, contrasted with lead citrate and examined in both laboratories with Philips CM–10 electron microscopes.

Controls

Both the monoclonal PA antibody and the polyclonal CB antiserum used in this study are well characterized and extensively used immunoreagents. However, to test the specificity of the immunostaining procedure, control experiments were performed. In these experiments, after replacing the primary antibodies with non-immune mouse or rabbit sera, no immunostaining could be observed.

Results

This work focused on the CA2 field of the primate hippocampus. However, other regions of the primate hippocampal formation were examined to determine the differences between the CA2 subfield and the other hippocampal areas. Detailed light and electron microscopic analyses of both parvalbumin- (PA) and calbindin- (CB) immunoreactive neurons located in other regions of the primate hippocampal formation will be provided in a future manuscript.

Anatomy of the CA2 subfield

A heterogeneous structure, named CA1, CA2, CA3, and CA4 by Lorente de Nó (1934), appears in frontal sections of the Ammon's horn. The morphological differences are largely due to the very different appearances of the pyramidal neurons in the different regions. The CA2 subfield is clear in human (Braak 1980) and simian hippocampi (Amaral et al. 1984), but is not readily identified in some non-primate species (Blackstad 1956; Schwerdtfeger 1984). The CA2 is composed of large, ovoid densely packed somata, making the stratum pyramidale dense and narrow, in sharp contrast to CA1 (Braak 1980). The other neighboring area of CA2, the CA3 subfield, corresponds to the curve, or genu of Ammon's horn where it enters the concavity of the dentate gyrus. Its pyramids are similar to those in CA2 but their density is less marked. The two zones are often distinguished by the presence of fine, unmyelinated fibers in CA3, the mossy fibers from the granule cells of the dentate gyrus. These fibers are in close proximity to the pyramidal somata of CA3 where they are compressed between the strata radiatum and pyramidale, thus forming a supplementary layer, the stratum lucidum (Duvernoy 1988).

Light microscopic observations

Immunostaining for CB. The pyramidal cells in the CA2 area exhibit a very strong immunoreactivity for CB (Figs. 1a and c). This dense pattern of CB-immunostaining within cell bodies and dendrites is also observed for the granule cells in the dentate gyrus (including their axons, the mossy fibers) and some nonpyramidal cells in the CA3 area (Fig. 1a). In contrast, the majority of CA1 pyramidal cells do not show immunoreactivity for CB, and those that do are located mostly in the upper half of the stratum pyramidale and are lightly stained compared to the CA2 pyramids. Similar to CA3, several densely labelled CB-immunopositive nonpyramidal neurons could be detected in the CA1 field. In contrast to the other subfields of the Ammon's horn of which all layers contain numerous CB immunoreactive nonpyramidal cells and fine varicose axons and boutons, the CA2 area

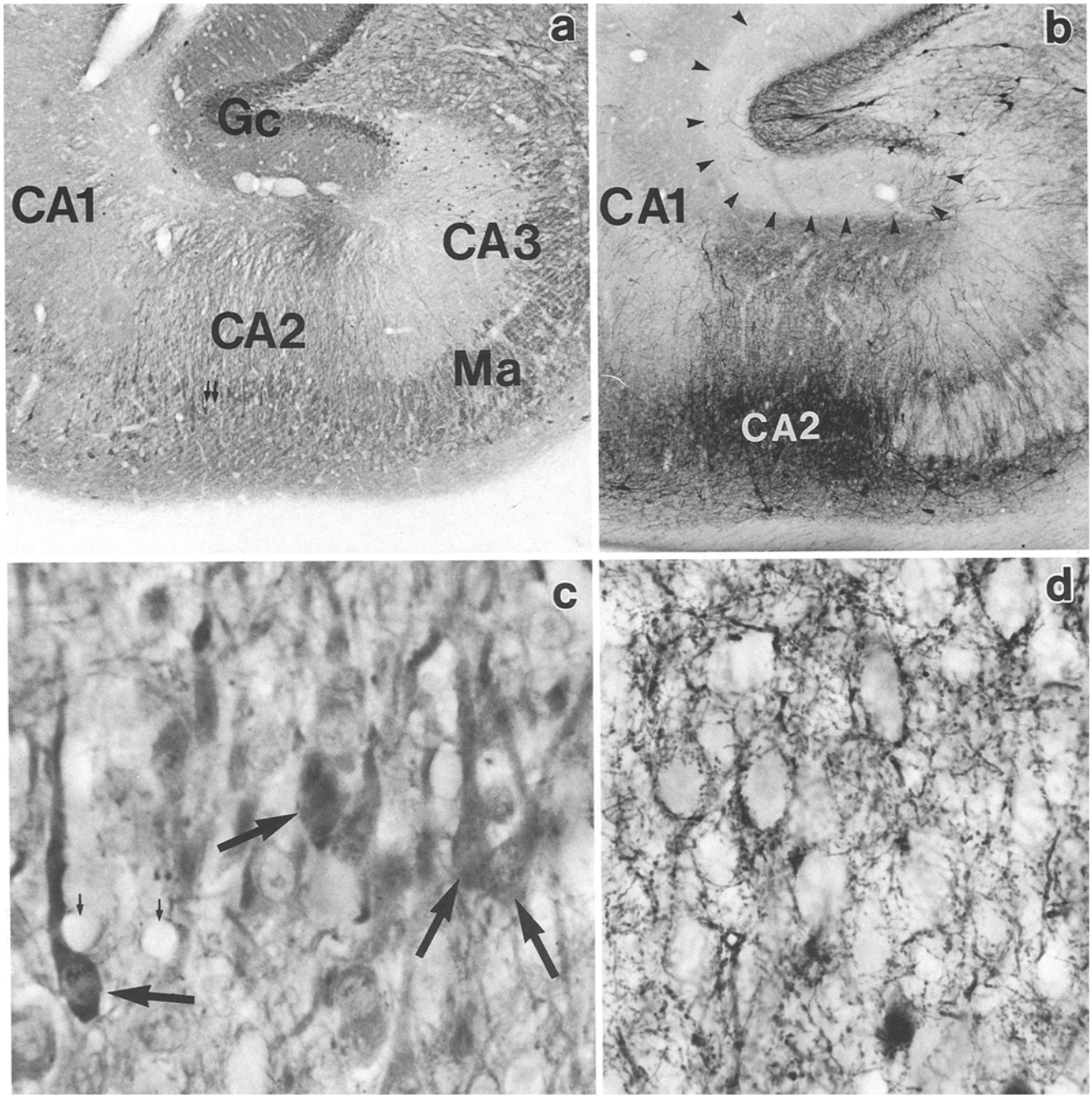

Fig. 1a–d. Light photomicrographs of 60 μm vibratome sections immunostained for calbindin (Panels **a** and **c**) and parvalbumin (Panels **b** and **d**) demonstrate identical areas of the primate hippocampal formation, including portions of the fascia dentata (outlined by arrowheads in Panel **b**), CA3, CA2 and CA1 areas. Panels **a** and **c** show that in addition to the heavy calbindin immunolabeling of the granule cells (Gc) and mossy axons (Ma), the CA2 pyramidal cells (some of the more intensively immunolabeled neurons located on the surface of the 60 μm vibratome section are identified by arrows in **c**) and apical dendrites are also calbindin-immunoreactive. Note that immunoreactivity for parvalbumin is the strongest in the CA2 area (Panel **b**). The parvalbumin-immunoreactive fibers form baskets around the cell bodies of CA2 pyramidal cells (Panel **d**) and also surround their dendrites. Small arrows on Panels **a** and **c** indicate the same blood vessels. Final magnifications are for Panels **a** and **b**: ×48; Panel **c**: ×480; and Panel **d**: ×1,000

contains only a few CB-immunopositive nonpyramidal neurons that are located chiefly in the stratum lacunosum-moleculare, and sparse fine varicose axons.

Immunolabeling for PA. In the primate hippocampal formation, the CA2 subfield displayed the greatest density of PA-immunostaining. Similar to other subfields of the Ammon's horn, the PA immunopositive cell bodies in CA2 are concentrated in the strata pyramidale and oriens. Many PA-positive dendrites in the stratum oriens run parallel to the pyramidal cell layer, whereas many other PA-positive dendrites pass through the stratum

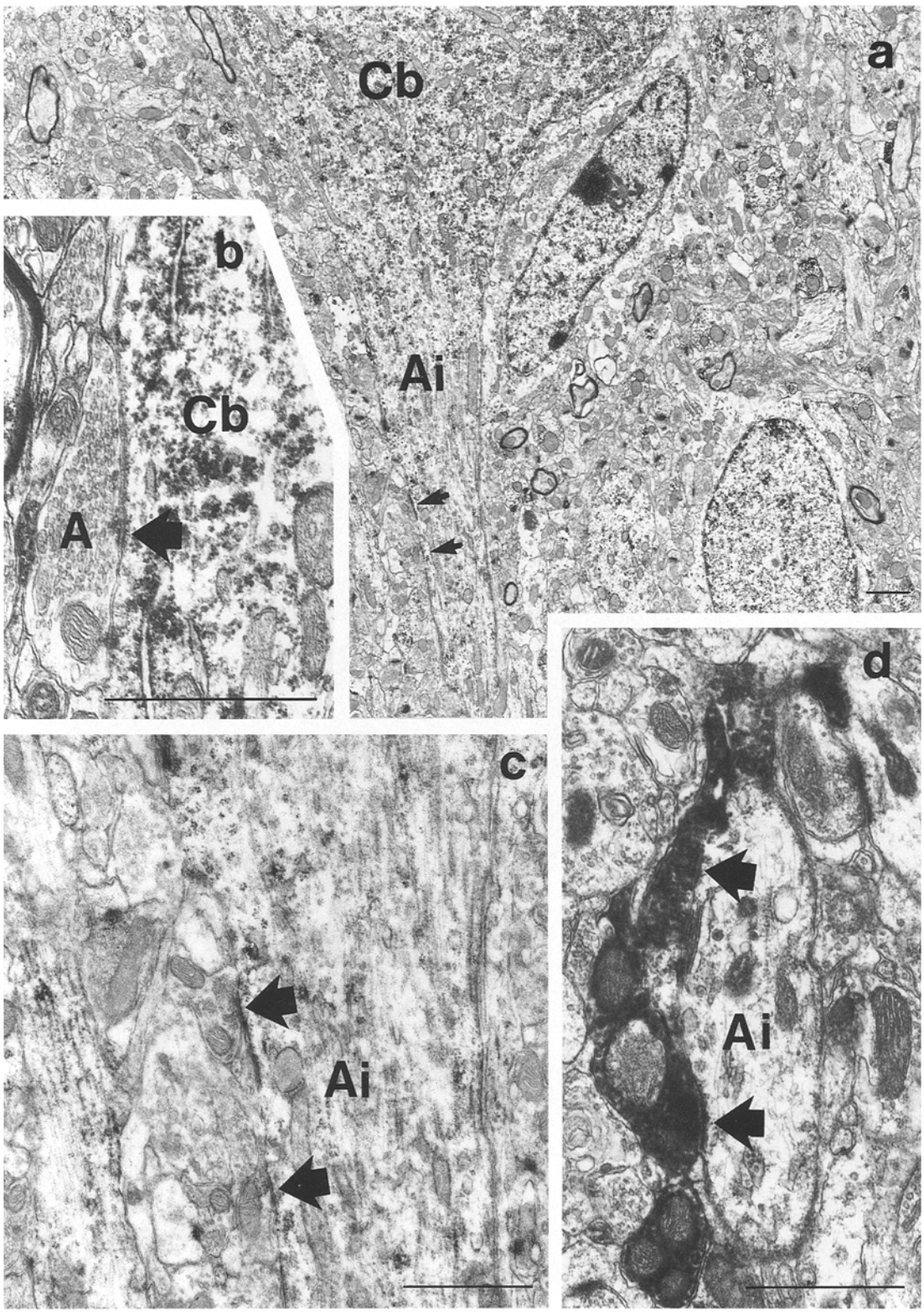

Fig. 2a–d. Electron micrographs taken from the monkey CA2 area show the result of immunostaining for calbindin (Panels **a–c**) and-parvalbumin (Panel **d**). Panels **a–c** demonstrate portions of a parvalbumin-immunoreactive pyramidal neuron. Panel a shows reaction product within a part of the cell body (CB) and axon initial segment (Ai) of this neuron. Panel **b** shows an axosomatic symmetric synapse (arrowhead) between an unlabeled bouton (A) and the immunostained cell body (CB). Panel **c** is an enlargement of the axon initial segment (Ai). Symmetric synapses (pointed by small and large arrowheads on Panels **a** and **c**, respectively) are formed with the axon initial segment (Ai) by unlabeled axon terminals. Note that the immunostaining is more intense in the cell body than in the axon initial segment. Panel d shows parvalbumin immunostained axon terminals forming symmetric synapses (arrowheads) with an immunonegative axon initial segment (Ai). Scale bars = 1 μm

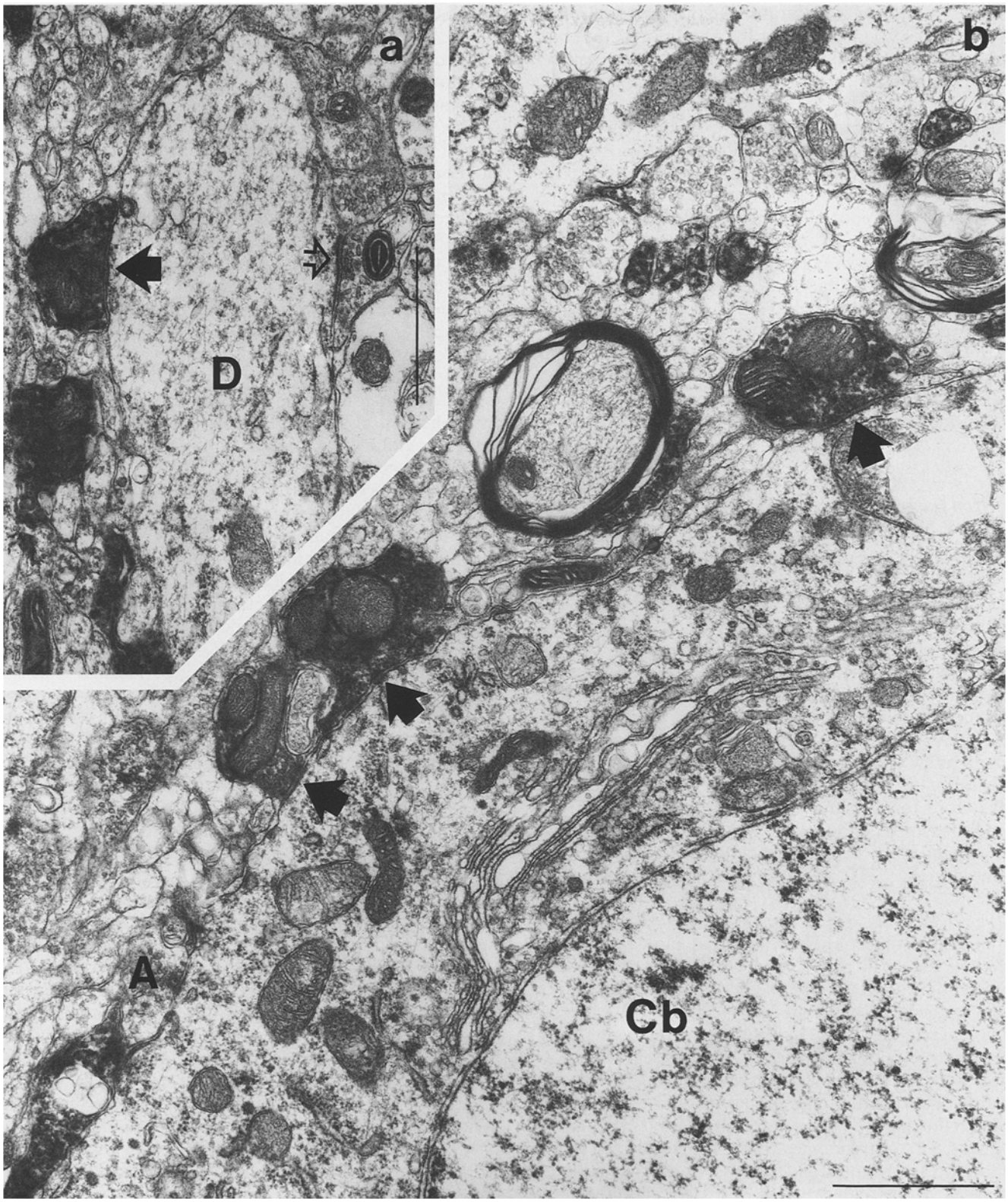

Fig. 3a, b. Electron micrographs taken from the parvalbumin immunostained primate hippocampus. Panel **a** shows a segment of an apical dendrite (D) of a pyramidal cell, whereas Panel **b** demonstrates part of a cell body (CB) of a CA2 pyramidal neuron. All of the synapses (black arrowheads) formed by parvalbumin immunoreactive boutons are symmetric. Note that only a few immunonegative boutons establish symmetric synapses, both with the dendrite (open arrow) and the soma (A), whereas the majority of synapses at these sites are formed by PA-positive axons. Scale bars = 1 μm

pyramidale and reach the stratum lacunosum-moleculare (Fig. 1b). The vast majority of PA immunoreactive axon terminals are densely packed in stratum pyramidale where the densely labeled boutons form basket like plexuses with the somata of pyramidal cells (Fig. 1d). Although PA-immunoreactive axonal baskets are present in the other CA subfields, the density of immunostaining in axons, and their number, appeared to be far less than that in CA2.

Another difference also exists between CA2 and the other CA subfields in the distribution pattern of PA-immunoreactive axons. In the CA2 area, a very dense network of PA-immunopositive axons and boutons is found in the stratum lacunosum-moleculare, whereas a less dense network is present in stratum radiatum. In other CA subfields the massive PA-immunostaining of the stratum lacunosum-moleculare is not present and the stratum radiatum displays a less dense pattern of PA immunoreactive axons (Fig. 1b).

Electron microscopic observations

For this study, electron microscopic results will only be reported for the CA2 area. The findings from this ultrastructural analysis of both CB- and PA-immunostained structures confirm the light microscopic observations.

Immunoreactivity for CB is homogeneously distributed throughout the cytoplasm of cell bodies and dendrites of CA2 pyramidal neurons. Furthermore, CB-immunoreaction product is also present in the axon initial segments of pyramidal cells (Figs. 2a and c). Although a few CB-immunoreactive axons are observed in the molecular layer of CA2, they appear to cluster in areas close to CA1 and CA3. A large number of CB-immunonegative axons form symmetric synapses with the somata (Fig. 2b), dendritic shafts, and axon initial segments (Fig. 2c) of CB-immunoreactive pyramidal cells.

The electron microscopic analysis of the PA-immunostained sections revealed that the vast majority of axons that establish symmetric synapses with dendrites (Fig. 3a), cell bodies (Fig. 3b), and axon initial segments (Fig. 2d) of pyramidal cells in CA2 are immunoreactive for PA. This finding correlates with the light microscopic observations of a dense PA-positive pericellular plexus in stratum pyramidale. Symmetric synapses between PA-immunonegative axons and pyramidal cell somata (Fig. 3b) are also found but are less frequent. PA-immunonegative axons formed both symmetric and asymmetric synapses with pyramidal cell dendrites. Consistent with the light microscopic data, several PA-immunopositive non-pyramidal neuronal somata and dendrites are present in the CA2 area. The dendrites and somata of these cells form symmetric synapses with PA-immunopositive boutons. In addition, these dendrites and somata display numerous asymmetric synapses with PA-immunonegative axons. PA immunoreactive neurons also display nuclei with deep infoldings, nuclear rods, and sheets.

Discussion

The major observation of this study is the highly concentrated distribution of calbindin (CB) and parvalbumin (PA), two important calcium-binding proteins, in the CA2 region of the primate hippocampal formation. Calcium overload is one of the major factors in the pathophysiology of hypoxic or excitotoxic cell damage (Desphande et al. 1987; Sloviter 1989). Recent studies (Scharfman and Schwartzkroin 1988) have shown that the injection of calcium chelators into neurons devoid of these two calcium-binding proteins protects these cells from deterioration during prolonged stimulation. Other experimental data suggest that hippocampal neurons equipped with calcium-binding proteins are protected against this calcium overload (Nitsch et al. 1989; Sloviter 1989), whereas some hippocampal neurons, such as somatostatin-containing cells that lack calcium-binding proteins (Nitsch et al. 1990a), are highly susceptible to hypoxia and overexcitation (Johansen et al. 1987, and 1989; Sloviter 1987).

In this respect, the CA2 subfield of Ammon's horn seems to be unique among the CA areas of the primate hippocampal formation. First, the CA2 pyramidal cells contain a very strong immunoreactivity for CB, whereas *none* of the CA3 pyramidal neurons did and only a few pyramidal cells located mostly in the upper part of the pyramidal cell layer in CA1 demonstrated a faint staining. Second, in comparison to CA1 and CA3 subfields, PA-immunopositive axons forming pericellular baskets around pyramidal cell somata in CA2 appeared more frequent and these axons, as well as those terminating on pyramidal cell dendrites exhibited a much stronger immunoreactivity. This more intense immunostaining may represent a greater concentration of PA. Finally, in contrast to other hippocampal regions, the CA2 area PA-immunoreactive boutons were found throughout the apical dendritic zone of the pyramidal neurons where they formed synapses (Fig. 3a). It remains to be shown where the cells of origin of these PA-immunoreactive boutons arise, especially those that give rise to the numerous axons in the outer stratum lacunosum-moleculae of CA2.

The PA-immunopositive axons that terminate on the pyramidal cell somata and axon initial segments, in all certainty, are processes of PA-containing basket- and chandelier cells, respectively (Nitsch et al. 1990; Ribak et al. 1990). The morphology of these axons is similar but the frequency of PA-positive boutons in CA2 of the monkey is greater than the 32–38% frequency observed in the rat hippocampal formation (Ribak et al. 1990). Recent studies have demonstrated that PA is colocalized with GABA in a population of nonpyramidal neurons (Celio 1986), including basket and chandelier cells (Kosaka et al. 1987; DeFelipe et al. 1989; Zipp et al. 1989; Soriano and Frotscher 1990). The structural characteristics of these neurons (Katsumaru et al. 1988; Nitsch et al. 1990b) are similar to those described in immunohistochemical studies of hippocampal GABAergic basket and chandelier cells (Ribak et al. 1981; Ribak and Seress 1983; Seress and Ribak 1990; Somogyi et al. 1985; Soriano and Frotscher 1989). Therefore, the PA-

positive axon terminals probably mediate GABAergic inhibition at their synapses.

The hippocampal basket and chandelier neurons are highly effective inhibitory neurons because their axon terminals are located at strategically optimal places to depress the firing rate of the CA2 area pyramidal cells. Although these inhibitory cells could be overstimulated during seizures, the presence of PA in high quantities (as detected with immunocytochemistry) may effectively protect them from dying. This view is supported by the observation that some GABAergic neurons are preserved in the hippocampus of animals with experimental epilepsy (Sloviter 1987), as well as in the human epileptic hippocampus (Babb et al. 1989; de Lanerolle et al. 1989).

In conclusion, the results of this study suggest that CA2 pyramidal cells are resistant to excitotoxicity from hyperstimulation due to their own protective calcium-binding protein content, as well as their very dense innervation with calcium-binding protein containing GABAergic inhibitory interneurons. This distinct chemical neuroanatomy in the primate CA2 area involving these two calcium-binding proteins, and perhaps others, may explain the relatively reduced vulnerability of CA2 area neurons in the temporal lobes of epileptic patients (Kim et al. 1990). Another factor that may also play a role in the resistance of CA2 pyramidal cells is their relative lack of excitatory afferents in that the mossy fibers, which innervate CA3, do not enter CA2 in primates (Houser et al. 1990), and the extent of Schaffer collaterals from CA3 cells may not be as large as that in CA1. Thus, not only may the chemical neuroanatomy of CA2 be responsible for its resistance to epileptic damage, but its relative lack of excitatory input may also be involved.

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