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Parvalbumin in Most γ-Aminobutyric Acid–Containing Neurons of the Rat Cerebral Cortex

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γ-Aminobutyric acid (GABA) is one of the major inhibitory neurotransmitters in the central nervous system. In the cerebral cortex, GABA-containing cells represent a subpopulation of interneurons. With semithin frozen sections, it is possible to demonstrate that most GABA neurons in the rat somatosensory cortex contain the calcium-binding protein parvalbumin and that parvalbumin is found virtually only in GABA neurons. Parvalbumin seems to influence the electrical properties and enzymatic machinery to modulate neuronal excitability and activity. The specific role of parvalbumin in GABA-containing cortical cells may be related to controlling the effectiveness of their inhibitory action.

THE CALCIUM-BINDING PROTEIN parvalbumin (PV) is a marker for a subpopulation of neurons (*1*). Calcium-binding proteins are low molecular weight, acidic proteins that buffer Ca^{2+}-ions or trigger the activity of various enzymes upon binding Ca^{2+} (*2*). Their presence may privilege a given neuron for certain Ca^{2+}-dependent processes. Since the cortical mantle displayed a large number of PV-immunoreactive local-circuit neurons, I sought to determine the relationship between PV- and γ-aminobutyric (GABA) interneurons in the rat somatosensory cortex.

Adult male albino rats were perfused transcardially with fixative, and small brain fragments were frozen in liquid nitrogen after imbibition with sucrose. Consecutive semithin (0.5- to 1-μm) sections were cut at −60°C with an ultracryomicrotome and mounted on glass slides (*3*). Adjacent sections were alternately incubated with PV-antiserum and GABA-antiserum. The primary antibody bound to the antigen was detected with the unlabeled antibody method and the peroxidase visualized histochemically (*4*). The methods currently available (legend to Fig. 1) made it possible to eliminate the danger of cross-reactions of the antisera used in immunohistochemistry.

In the 0.5- to 1-μm-thick cryo-sections, the GABA and PV immunolabelings were closely matched (Fig. 2, A and B). Two different interneuronal populations were revealed: the major population (approximately 70 percent) contained both GABA and parvalbumin (GABA-plus-PV neurons), whereas a minor population (approximately 30 percent) had only GABA immunoreactivity (GABA-only neurons). With few exceptions, parvalbumin distributed itself only in GABA cells (Fig. 2). In the sections incubated with the GABA-antiserum (Fig. 2A), the cell body and the nuclei were homogeneously immunostained. The neuropil was abundant with immunoreactive ter-

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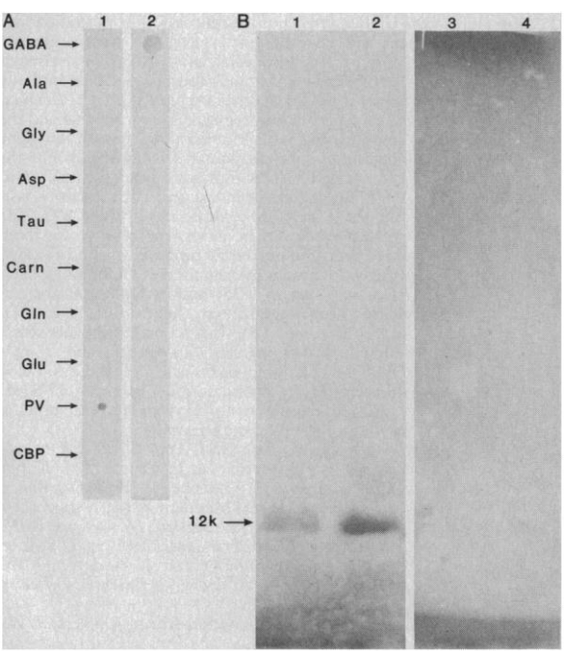

Fig. 1. Specificity controls for the immunoreaction. (A) The specificities of the GABA and PV antisera were tested by spotting possibly crossreacting substances on glutaraldehyde-activated nitrocellulose paper (*5*). A 2-μl portion of a 200-m*M* solution of following amino acids was applied: GABA; Ala, β-alanine; Gly, glycine; Asp, aspartate; Tau, taurine; Carn, carnosine; Gln, glutamine; Glu, glutamate (all from FLUKA, Switzerland). The calcium-binding proteins PV and calbindin (CBP) were applied in concentrations of between 0.01 and 1 mg/ml. In this system, which mimics immunohistochemistry, the PV antiserum reacted only with PV (lane 1), whereas the GABA-antiserum recognized only GABA (lane 2) at concentrations which may be assumed to occur in the tissue. The GABA antiserum, however, reacted with β-alanine at high concentrations of this substance spotted on nitrocellulose. The antisera were also adsorbed with the homologous and heterologous antigen as well as with bovine calmodulin (FLUKA) and β-alanine bound to polyacrylamide beads with glutaraldehyde (*5*).The immunostaining with the PV antiserum was inhibited only by the PV conjugate, while the GABA-immunostaining was inhibited only by GABA. (B) In an alternative specificity test (*15*), the cerebellum of a rat killed with an overdose of anesthetics was homogenized and subjected to sodium dodecyl sulfate–gel electrophoresis, subsequently transferred to a sheet of nitrocellulose paper, treated with glutaraldehyde, and incubated with the PV or the GABA antiserum. Cerebellar extract (10 μg) (lanes 1 and 3) and 10 μg of purified rat muscle parvalbumin (lanes 2 and 4) were run in parallel, blotted to the nitrocellulose paper, incubated with the PV (lanes 1 and 2) or the GABA (lanes 3 and 4) antisera, and reacted with an indirect peroxidase method. The PV antiserum used in this study recognized a protein which displayed the same electrophoretic mobility as purified muscle PV (12K). The GABA antiserum did not cross-react with any cerebellar proteins. Lanes 1 and 2 were left for 15 minutes and lanes 3 and 4 for 1 hour in the peroxidase substrate solution.

minals, which often abutted on unlabeled cell bodies, most probably from pyramidal neurons, and sometimes also on GABA-immunoreactive neurons. After incubation with the PV antiserum (Fig. 2B) the same perikarya and most of the same terminals in the neuropil exhibited immunoreactivity. GABA-plus-PV perikarya were generally round or fusiform and varied in diameter between 15 and 25 μm. GABA-plus-PV neurons were found in all cortical layers except layer I. The GABA-only neurons were found in the whole thickness of the cerebral cortex but were preferentially associated with layers I and II.

The somatosensory cortex is not the only region with neurons in which GABA and PV coexist. Most neurons in the rest of the cortical mantle, all neurons of the reticular nucleus of the thalamus and of the pars reticulata of the substantia nigra, as well as the basket cells of the hippocampus and the Purkinje, basket, and stellate cells of the cerebellum, were positive for both GABA and glutamic acid decarboxylase (*5*) and for PV (*1, 6*). On the other hand, there are known GABA-ergic systems, for example the striato-nigral projection and the cerebellar Golgi cells, which do not contain parvalbumin. Although PV is found almost exclusively in GABA cells in cortical regions, it is also found alone in other areas of the central nervous system, including the afferents from the retina and the organ of Corti (*1, 6*) which are not thought to contain GABA.

Calcium ions and GABA play important, although independent, roles in the control of neuronal excitability. The results of this study associate in the same neuron the calcium-modulating protein PV and the inhibitory neurotransmitter GABA. This suggests a previously unsuspected importance of mechanisms related to Ca^{2+} or Mg^{2+} for the activity of most, but not all, GABA neurons.

The role of the calcium-binding protein PV in GABA cells must be related to a characteristic of most of these cells that differentiates them from other neurons (*7*). Most GABA cells can be singled out by at least three different criteria. Electrophysiologically, GABA neurons in the cerebral cortex, hippocampus, cerebellum, and nucleus reticularis thalami are characterized by their high firing rates (*8*). As a consequence, GABA neurons are very active metabolically and contain high concentrations of the mitochondrial enzyme cytochrome oxidase (*9*). Furthermore, most GABA neurons receive mainly excitatory afferents, which dominate the dendritic and somatic surface (*10*).

Is there any causal relation between the presence of PV and the expression of these three characteristics of GABA neurons? If it can be assumed that the biochemical role of PV in the brain is to bind Ca^{2+} in exchange for Mg^{2+}, as is true in skeletal muscles (*11*), PV may be able to affect the excitability and the activity of GABA neurons. By binding Ca^{2+} after its entry into the cytoplasm, PV would prevent the activation of the Ca^{2+}-activated K^+ current (*12*), thus reducing the afterhyperpolarization and accommodation of the neuron. The shortening of the refractory period would enable the neuron to fire in rapid repetition and to recover more quickly from a presynaptic excitatory bombardment. At the same time, by discharging Mg^{2+} in the cytoplasm, PV may trigger the multitude of Mg^{2+}-dependent enzymes (*13*) thus linking cell activity and cell metabolism. Thus, PV may be thought of as a common denominator underlying the three distinctive characteristics of most GABA neurons.

The notion that PV confers on neurons the ability to be electrically and metabolically more active than companion neurons is indirectly supported by the fact that the striato-nigral projection neurons and the cerebellar Golgi cells—representatives of the GABA-positive but PV-negative neurons—are metabolically less active and fire at low rates (*14*).

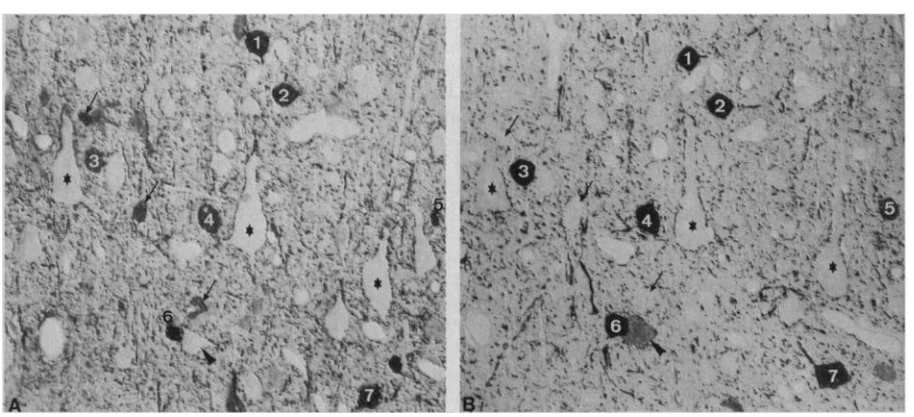

Fig. 2. Immunohistochemical detection of the "inhibitory" neurotransmitter GABA and of the calcium-binding protein PV on two consecutive semithin (0.5-μm) sections of the cerebral cortex of the adult rat brain. (A) GABA-immunoreactivity was found in seven neurons scattered in layers IV and V of the rat somatosensory cortex. (B) On a consecutive section the same neuronal cell bodies reacted with the PV antiserum. Exceptions to this rule were encountered; in these cases the GABA cell has no PV (arrow) and, very rarely, the PV cell has no GABA (arrowhead). Note the differences in staining intensity between PV neurons and the richness of terminals in the neuropil and around pyramidal cells somata. The asterisk marks three unreactive pyramidal cell bodies as landmarks. The background staining is higher in the section immunoreacted with GABA ×100; Nomarsky optics.

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16. This report is dedicated to Dr. Rafael Lorente de Nò on the occasion of his 83rd birthday. I thank L. Schärer and G. Weinbrenner for their excellent technical assistance; H. Haas for his continuous help and interest; P. Somogyi who kindly supplied me with preprints of his papers; P. Streit and C. Matute for suggestions concerning the cross-reaction tests; and C. W. Heizmann, J. M. Gillis, P. Sequela, and A. W. Norman for their gift of antisera and antigens. Supported by Swiss National Foundation 3.559.0.83 and Hartmann-Müller-, Mobiliar-, Rentenanstalt-, Sandoz- and EMDO-Stiftung.

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Tripeptide Structure of Bursin, a Selective B-Cell–Differentiating Hormone of the Bursa of Fabricius

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Differentiation of lymphoid precursor cells in a variety of species is induced by polypeptide hormones such as thymopoietin for T cells and bursin for B cells. In the present experiments, bursin isolated from the bursa of Fabricius of chicken was found to induce the phenotypic differentiation of mammalian and avian B precursor cells but not of T precursor cells in vitro. Similarly, bursin increased cyclic guanosine monophosphate in cells of the human B-cell line Daudi but not in cells of the human T-cell line CEM. These inducing properties of bursin are the reverse of the inducing properties of thymopoietin produced by the thymus and are appropriate to a physiological B-cell–inducing hormone. A tripeptide sequence (lysyl-histidyl-glycylamide) was determined for bursin and confirmed by synthesizing this proposed structure and demonstrating chemical identity of the natural and synthetic peptides. Similarity of biological action was indicated in induction assays by elevation of cyclic adenosine monophosphate and guanosine monophosphate in Daudi B cells but not in CEM T cells.

EARLY IN DEVELOPMENT, TWO SEPARATE differentiational pathways for lymphocytes are established, one for T (thymic) lymphocytes and the other for antibody-secreting B (bone marrow and bursal) lymphocytes. Throughout life, committed precursor T cells (prothymocytes) migrate from the bone marrow to the thymus, where they differentiate further under the influence of the thymic hormone thymopoietin (*1*). This induction of prothymocytes is assayed in vitro by measuring the appearance of thymocyte phenotypic markers in serum, which occurs within a few hours and is accompanied by elevation of intracellular adenosine 3′,5′-monophosphate (cyclic AMP) (*2*). Thymopoietin has later immunoregulatory actions [mediated by intracellular guanosine 3′,5′-monophosphate (cyclic GMP) elevation] on more mature T cells (*3*). These biological actions of thymopoietin are reproduced by the synthetic pentapeptide thymopentin (Arg-Lys-Asp-Val-Tyr) (*4*). Thymopoietin and thymopentin selectively induce differentiation of prothymocytes but not of precursor B cells (*2*). This inductive selectivity distinguishes thymopoietin from other prothymocyte inducers, which show neither this selectivity nor limitation of production to the thymus.

In mammals, no B-cell–differentiating organ equivalent to the T-cell–differentiating thymus has been defined, and the existence of a mammalian B-cell–differentiating hormone equivalent to thymopoietin has not been unequivocally demonstrated. In birds, however, B-cell differentiation occurs within the bursa of Fabricius, a dorsal diverticulum of the cloaca (*5*). Bursal extracts contain the low molecular weight inducing agent bursin (formerly termed bursopoietin) (*6*). Bursin selectively induces avian B cells, but not avian T cells, from their precursors in vitro (*6*). Elevation of intracellular cAMP accompanies this inductive process in the chicken, as in the mammalian lymphocyte induction assays. In both the mouse and chicken, avian bursin selectively induces B cells but not T cells (*7*). We now report that bursin elevates cyclic AMP and cyclic GMP in human cells of the Daudi line, derived from a Burkitt's lymphoma, and we have used this observation as an assay for bursin. For tests of inductive selectivity we used the inducible human T-cell line CEM in parallel.

Frozen chicken bursa (80 g, Pel-Freeze Biologicals) was homogenized with 800 ml of 1*N* acetic acid at 4°C in a Waring blender. After centrifugation at 14,000*g* for 60 minutes at 4°C, the supernatant was removed and recentrifuged. This supernatant was evaporated in a Rotavapor-RE and lyophilized, and the lyophilizate was dissolved in 1*N* acetic acid at room temperature for 1 hour and centrifuged at 12,000*g* for 20 minutes. This supernatant was further frac-

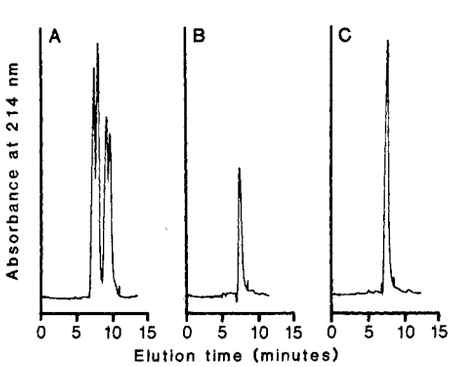

Fig. 1. Chromatographic comparisons of native bursin and four synthetic tripeptides. HPLC was performed on an Analyst series 7800 HPLC system with a Constametric III pump and Zorbax octadecyl silane analytical column. Peptides were eluted with 200 m*M* perchloric acid at 0.5 ml/min and the elution was monitored at 214 nm. (A) Coinjection of Lys-His-Gly-NH_2 (7.3 minutes), Gly-His-Lys-NH_2 (7.9 minutes), Lys-His-Gly (9.1 minutes), and Gly-His-Lys (9.6 minutes). (B) Injection of native bursin (7.3 minutes). (C) Coinjection of equal amounts of native bursin and Lys-His-Gly-NH_2, showing a single peak with a retention time of 7.3 minutes.

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