

# Immunocytochemical localization, in synapses, of protein I, an endogenous substrate for protein kinases in mammalian brain

(cyclic AMP/synaptic vesicles/synaptic membranes/light microscopy/electron microscopy)

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**ABSTRACT** Protein I is a principal endogenous substrate for cyclic AMP- and  $\text{Ca}^{2+}$ -dependent protein kinases of mammalian brain. Antibodies raised against purified protein I have been used to localize this protein in the rat central nervous system. At the light microscope level, immunoreactivity was detected in punctate deposits in selected zones of synaptic termination. These deposits varied in density among brain regions. At the electron microscope level, immunoreactivity was observed at some but not all synaptic regions and was restricted to the perimeter of synaptic vesicles and to submembranous material in the postsynaptic neuron.

Evidence is accumulating to indicate that many intracellular regulatory agents achieve certain of their biological effects through regulation of protein phosphorylation. In the nervous system, cyclic AMP-dependent, cyclic GMP-dependent, and  $\text{Ca}^{2+}$ -dependent protein kinases have been demonstrated, and endogenous substrate proteins for each of these three types of protein kinase have been found (1). One protein, which is a prominent substrate both for cyclic AMP-dependent and  $\text{Ca}^{2+}$ -dependent protein kinases in the mammalian central nervous system, has been referred to as protein I (2, 3). This protein is composed of two types of polypeptides with apparent molecular weights of 86,000 (protein Ia) and 80,000 (protein Ib) (4). Protein I appears simultaneously with synapse formation during development of the mammalian brain (5); in brain slices (6) and in whole animals (7) its state of phosphorylation is affected by agents that affect the physiological state of nerve cells.

In the present investigation, antibodies raised against purified bovine brain protein I have been used to locate this protein in immunocytochemical studies of the rat brain. The accompanying paper presents results of immunocytochemical studies of protein I outside the brain (8). The combined results of these studies indicate that protein I is localized to neurons and that, within neurons, protein I is localized to synaptic vesicles and subjunctional postsynaptic membranes of certain central and peripheral junctions. These results on the immunocytochemical localization of protein I are in agreement with results of subcellular fractionation studies reported elsewhere (9).

## MATERIALS AND METHODS

**Preparation of Immunogen Protein and Antisera.** Protein I was purified to apparent homogeneity from bovine brain as described (4). Antibodies to purified protein I were raised in a New Zealand female rabbit. One week prior to the injection, the rabbit was bled to obtain preimmune serum. Protein I, 200  $\mu\text{g}$  emulsified in complete Freund's adjuvant, was injected intradermally at multiple sites at 0, 2, and 4 weeks. The rabbit was bled at 6 weeks and biweekly thereafter. Data establishing

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the specificity of the anti-protein I antiserum are reported in the accompanying paper (8).

**Preparation of Tissue and Immunocytochemical Reactions.** For immunocytochemical studies at the light microscope level, rats were anesthetized and perfused and brain tissues were prepared exactly as described for the localization of  $\beta$ -endorphin (10). Immunocytochemical studies were accomplished with four different protocols, each involving indirect procedures to detect structures that bound the anti-protein I antiserum used either as whole serum or as the IgG fraction. The preimmune and immune sera were diluted in phosphate-buffered saline containing 0.3% Triton X-100 (Sigma) and 1 mg of bovine serum albumin (McAllister-Bicknell, New Haven, CT) per ml.

**Protocol 1.** This was a two-step indirect immunofluorescence method with the primary antiserum used in 1:50 to 1:100 dilution for incubations of 30–500 min at 4°C and fluorescein isothiocyanate-conjugated goat immunoglobulin raised to rabbit immunoglobulin (Cappel, Cochranville, PA) at 1:50 dilution at room temperature for 30 min. After washings, specimens were coverslipped with Entellan or paraffin oil and examined in a Zeiss Universal microscope with epi-illumination through the fluorescein isothiocyanate filter pack.

**Protocol 2.** This was a two-step indirect immunoperoxidase method with the primary antiserum at 1:1000 dilution and a goat IgG raised against rabbit IgG to which Jean Rossier (Roche Institute, Nutley, NJ) conjugated horseradish peroxidase (10, 11), used at 1:1000. In this case, incubations were for 12–14 hr at 4°C in the primary antiserum and for 60 min at room temperature in the secondary antiserum.

**Protocol 3.** This three-step unlabeled indirect antibody method, as described by Sternberger (12), used unlabeled goat IgG (1:100) and rabbit peroxidase-antiperoxidase complex (1:50) (both from Cappel) in two consecutive steps separated by extensive washes after incubation of sections in the primary antiserum as defined in protocol 2 as described (10).

**Protocol 4.** This was a four-step unlabeled indirect method, as described by Mason *et al.* (13), that used the primary antiserum at 1:1000, unlabeled goat antirabbit IgG at 1:100, unlabeled rabbit antiperoxidase at 1:100 (both from Cappel), and, finally, horseradish peroxidase (Sigma; 1.25 mg/100 ml in phosphate-buffered saline), each applied consecutively after extensive rinses as described (10).

In each case the horseradish peroxidase was developed as reported (10).

Distribution of immunoreactive structures was defined neuroanatomically according to standard rat brain atlases (14, 15). All results have been replicated a minimum of six times in different animals.

For electron microscopic localization, methods were largely restricted to protocols 2 and 4 above. Segments of brain regions to be examined were first cut on a vibratome (Oxford Instruments, San Mateo, CA) at 30–50  $\mu\text{m}$ , at 4°C and incubated as

free-floating sections in small plastic petri dishes in the primary and subsequent immune antisera and washing solutions, according to the times and dilutions used for light microscopy. Departures from the protocols occurred only at the stage of developing the peroxidase marker: sections were preincubated in 0.05% diaminobenzidine tetrahydrochloride (Sigma) in 0.05 M phosphate buffer (pH 7.4) at 4°C for 30 min. The sections were then incubated for 10–20 min in freshly prepared 0.05% diaminobenzidine in the same buffer containing 0.003% H<sub>2</sub>O<sub>2</sub>. Sections were then rinsed in phosphate-buffered saline or Ringer's solution and postfixed in either 1% OsO<sub>4</sub> in 0.15 M phosphate buffer or 4.5% KMnO<sub>4</sub> (in Ringer's solution) for 30 min at 4°C. Sections fixed in KMnO<sub>4</sub> were then rinsed briefly in Ringer's solution and block stained for 1 hr at 4°C with 4% uranyl acetate in Ringer solutions (Upjohn). Tissues were then dehydrated in progressively more concentrated ethanol solutions, embedded in epoxy resin (TAAB), and prepared for examination by electron microscopy (Zeiss EM 10).

In the immunocytochemical procedures, specificity of immunoreactivity with anti-protein I was demonstrated by comparison with tissues treated with control sera (naive rabbit serum and preimmune serum from the immunized rabbit) and with anti-protein I antisera to which a 10-fold saturating amount of purified protein I was added 18 hr before reaction with tissue sections. Specificity was also demonstrated by comparison with staining patterns obtained with a large number of other antisera raised to specific glial or neuronal components including [Leu]enkephalin, somatostatin, substance P, neurotensin, leuteinizing hormone releasing factor,  $\beta$ -endorphin, and corticotropin (results not shown). All staining described here as attributable to anti-protein I was eliminated by prior absorption with the purified protein I immunogen. There was no staining of brain tissue when preimmune serum or serum from naive animals was used in our protocols.

## RESULTS

Specific immunoreactivity for protein I was found to be restricted to fine punctate structures that surrounded neuronal perikarya and that were especially enriched in certain neuropil fields (Fig. 1). Using the highly selective distribution of immunoreactivity detected in cerebellar cortex, we evaluated several fixation, sectioning, and incubation procedures to develop the optimal protocols described here. As with localization of  $\beta$ -endorphin (10), optimal immunoreactivity for protein I was obtained in brains fixed by perfusion with 4–5% depolymerized paraformaldehyde; some distinct and specific immunoreactivity was retained after addition of 0.25% glutaraldehyde to this fixative, but no higher concentrations of glutaraldehyde were compatible with full immunoreactivity. The two-step indirect immunoperoxidase method provided the cleanest reaction with minimal background immunostaining; the unlabeled four-step method (13) gave slightly improved penetration into the incubated sections.

Specific immunoreactivity to protein I was detected in every region of the nervous system but in patterns and intensities compatible with a highly selective distribution of this protein. Staining reactivity occurred as punctate deposits (0.2–5  $\mu$ m in diameter; see Fig. 1 *a, c–g*) which were found in zones of neuropil and at the perimeter of distinct neuronal perikarya (Fig. 1 *c–e*). No anti-protein I immunoreactivity was detected inside any neuron at the light microscopic level of resolution, nor was there any immunoreactivity associated with white matter, blood vessels, meninges, or ependyma.

The pattern of punctate staining was quite intense within the molecular layer of the cerebellar cortex, the caudate nucleus, the hilus of the dentate gyrus and adjacent fields CA3 and CA4

of the hippocampal formation, within the external plexiform and mitral cell layers of the olfactory bulb, and within the external lamina of the median eminence. Some degree of staining was present in the neuropil of every brain region examined but, in relative terms, cerebral cortex and most diencephalic nuclei were less reactive than the areas named above. The distinctive nature of specific staining patterns suggested that cerebellar mossy fibers (Fig. 1 *a* and *b* and Fig. 2*a*) and hippocampal mossy fibers in fields CA3 and CA4 (Fig. 1 *d* and *e*) were reactive. However, in most cases it was not possible to identify with certainty the type or class of immunoreactive nerve terminal observed by light microscopy. In rare cases, such as the median eminence (Fig. 1 *f* and *g*), deep cerebellar nuclei, and brainstem, the punctate deposits were linearly aligned and apparently connected much as the nerve fibers which, with other antisera, are immunoreactive for certain neurotransmitters (see ref. 10).

Observations by electron microscopy (Fig. 2) were restricted to the moderately reactive zones lying just below the outermost surfaces of the incubated Vibratome sections of cerebellum and hippocampus. In these zones, clear-cut immunoreactive structures could be identified. At the ultrastructural level, immunoreactivity was observed in some synaptic terminals but not in all; when present in the terminals, the immunoprecipitates were located at the perimeter of the synaptic vesicles (Fig. 2 *a* and *b*). When immunopositive synaptic terminals were sectioned so as to reveal the postsynaptic structure, the electron density of submembranous material at this site was distinctly enhanced over that of control sections (Fig. 2 *b* and *c*) and over that of the postsynaptic membrane material in unreactive junctions within the immediately adjacent zone of the same fields exposed to the anti-protein I antiserum (see Fig. 2*a*). No immunoreactive postsynaptic membrane elements were observed in which the presynaptic terminal was unreactive.

In other immunocytochemical experiments, no enhanced immunoreactivity of postsynaptic membrane has been seen with our protocol when antisera to neuropeptides yielded intense staining of presynaptic terminals (results not shown). In some cases of symmetrical synaptic junctions [in which postsynaptic paramembranous electron density is normally minimal (16)] the amount of enhanced electron density visualized on the postsynaptic side was also minimal. The use of KMnO<sub>4</sub> as the postreaction fixative provided the opportunity to achieve a greater degree of contrast of the plasma membranes and diminished the basic electron density associated with specialized synaptic contact zones; under these conditions the enhanced electron density due to protein I immunoreactivity was more readily distinguished from controls, especially for those terminals, such as cerebellar mossy fibers, that could be identified most confidently (Fig. 1 *a* and *b*) (17). In intensively reactive presynaptic boutons, some enhanced electron density could be seen at the perimeter of mitochondria within the terminal. This staining may have been due to diffusion from the immunoreactive synaptic vesicle surfaces, because immunoprecipitates on mitochondrial perimeters were not invariably observed within reactive terminals. Furthermore, when present, these mitochondrial immunoprecipitates were always most pronounced on those surfaces closest to the vesicles.

We cannot exclude the possibility that diffusion of protein I, or of immunoprecipitate, is responsible for the apparent immunoreactivity of material beneath the postsynaptic membrane. However, in favorably sectioned junctions studied at high magnification, the enhanced electron density was found to be specific for the internal (cytoplasmic) surface of the presynaptic membrane without enhancement of the external surface of that membrane; on the postsynaptic side, both sur-

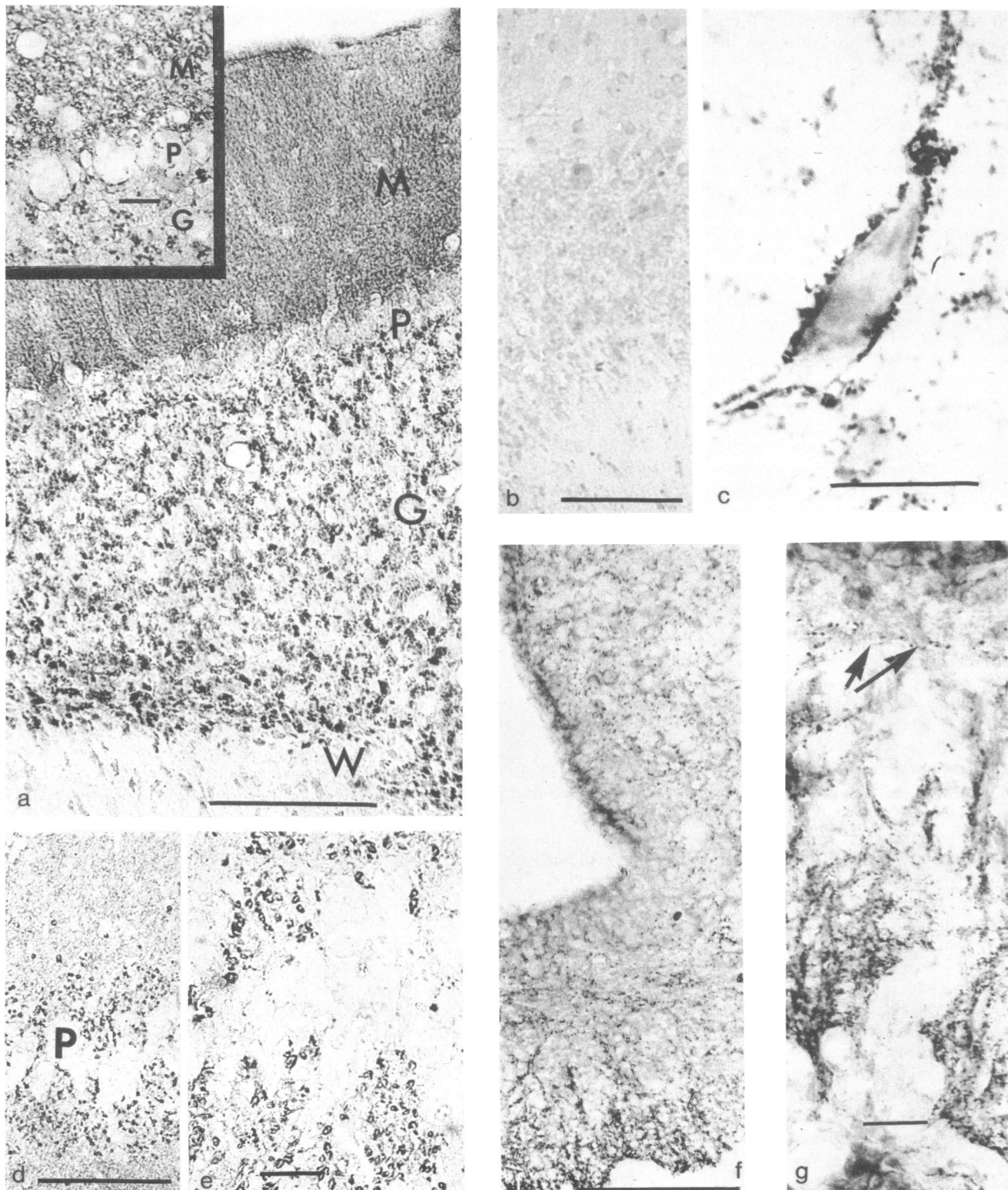


FIG. 1. Immunoperoxidase reactivity of rat brain regions prepared by protocol 2 with anti-protein I antiserum and sections through cerebellar cortex (*a* and *Inset*), nucleus reticularis thalami (*c*), and CA3 region of hippocampus (*d* and *e*). No immunoreactivity was seen when preimmune serum was substituted for anti-protein I antiserum (*b*). In each reactive region, small punctate reactive elements can be seen surrounding neurons (*a Inset* and *c*), in the molecular (M) and granular (G) layers of the cerebellar cortex (*a*), and above and below the pyramidal (P) cell layers of the hippocampus (*d* and *e*). No staining was observed with the perikarya of Purkinje (P), pyramidal (P), or other neurons nor in white matter (W). (Calibration bars: *a*, 100  $\mu$ m; *Inset*, 20  $\mu$ m; *b*, 50  $\mu$ m; *c*, 25  $\mu$ m; *d*, 100  $\mu$ m; *e*, 20  $\mu$ m.)

(*f* and *g*) Median eminence prepared for anti-protein I immunoperoxidase reaction by protocol 2, showing intense clusters of immunoreactive elements in the superficial external zone of this structure (bottom) with substantially decreased density of immunoreactivity in the internal zone and in portions of the hypothalamus adjacent to the third ventricle. Linearly aligned reactive elements suggestive of boutons *en passage* within a single nerve fiber can be seen at higher magnifications in the internal zone (arrows in *g*). (Calibration bars: *f*, 100  $\mu$ m; *g*, 20  $\mu$ m.)

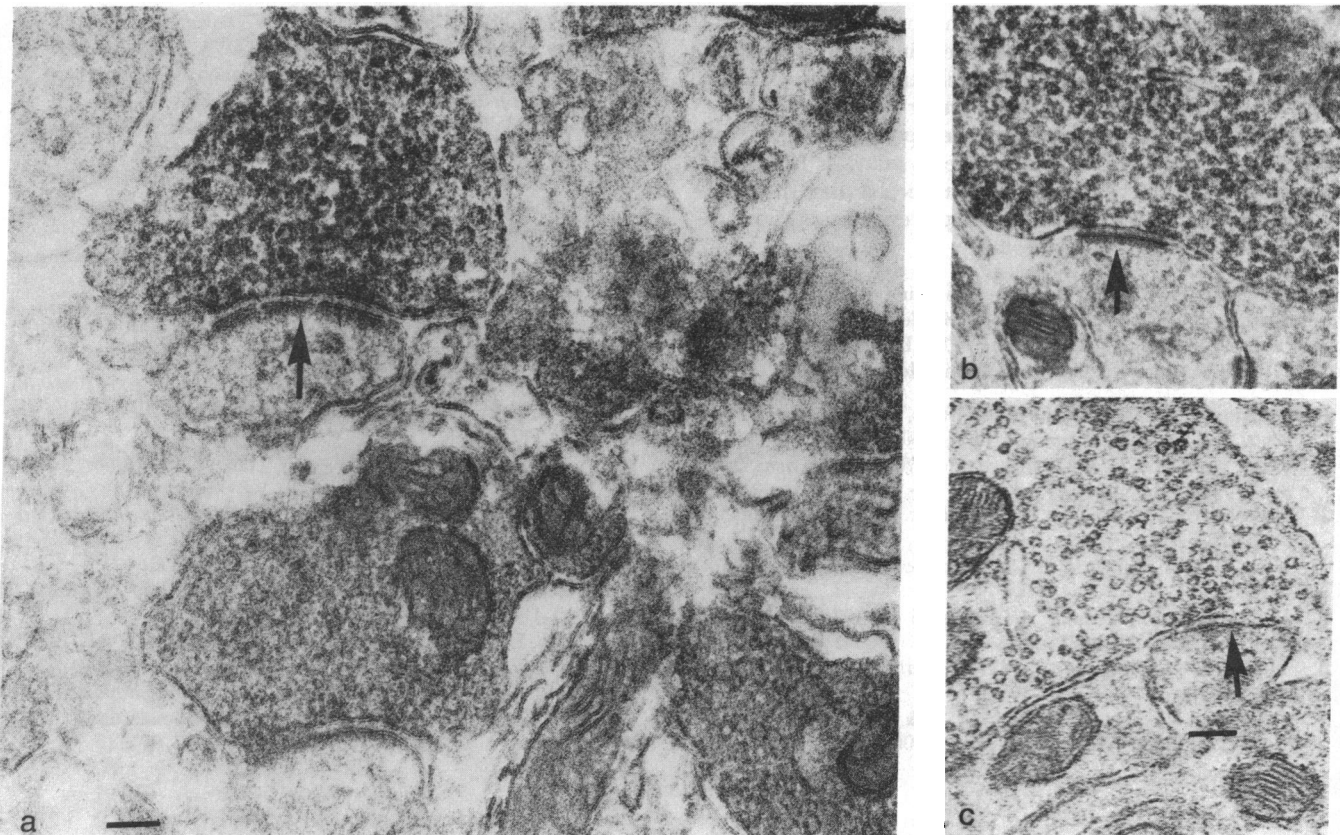


FIG. 2. Electron microscopic localization of protein I immunoreactivity in cerebellar terminals stained with immune serum (*a* and *b*) by protocol 2, followed by  $\text{KMnO}_4$  fixation. Immunoprecipitates are observed within nerve terminals on the perimeter of the synaptic vesicles and within the postsynaptic membrane specialization (arrows). No immunoreactivity is seen when preimmune serum is substituted for the immune serum (*c*), and, with sections of comparable thickness, sites of apparent synaptic specialization inferred from presynaptic clusters of vesicles show only minimal to modest electron density of the postsynaptic specialization. (Calibration bars: *a*, 0.1  $\mu\text{m}$ ; *b* and *c*, 0.1  $\mu\text{m}$ .)

faces as well as submembranous material were enhanced in electron density, indicating immunoreactivity.

In cerebellar cortex, and in limited examinations of the hippocampal cortex in area CA3, it was apparent from electron microscopy that only a small fraction of synaptic terminals stain for protein I. Terminals in cerebellar cortex with the appearance of parallel fibers, and representing perhaps the largest single class of terminals in the molecular layer (17), were the most prominent variety of unreactive terminal.

#### DISCUSSION

Immunocytochemical studies with antiserum developed against purified protein I indicate that specific neuronal structures associated with synaptic junctions are the major locus of this protein. Such synaptic terminals represented a small fraction of all terminals present, even in those areas in which the most intense immunoreactivity was observed. No staining was apparent within neuronal perikarya by light microscopy, and no non-neuronal elements could be found to exhibit immunoreactivity.

By electron microscopy, immune staining was largely found within presynaptic structures, where it was associated with the perimeter of the synaptic vesicles, the internal surface of the presynaptic plasma membrane, and, on occasion, the external surface of the mitochondria. In some synaptic junctions, the postsynaptic plasma membrane at the junctional point was enhanced in electron density on both surfaces, and submembranous material also appeared to be immunoreactive, compared to the postsynaptic electron density of specialized junctional zones for unreactive synapses.

This distribution of immunoreactivity is also compatible with results of subcellular fractionation studies (9) and with the developmental appearance of protein I (5). The synaptic location of protein I is suggestive of its involvement in junctional mechanisms. The immunoreactivity of presynaptic vesicles could reflect some aspect of cyclic nucleotide- or  $\text{Ca}^{2+}$ -mediated regulation of transmitter synthesis, storage, or release (18, 19). However, the present inability to establish the transmitter within the immunoreactive terminals limits this line of interpretation. The presence of anti-protein I immunoreactive terminals in regions known to be rich in catecholamines, such as the hilus of the dentate gyrus in the hippocampus (20, 21) and the caudate nucleus (22), is not an adequate explanation for the distribution of protein I because the density and distribution of catecholamine terminals cannot explain the occurrence of reactive material in some other brain regions. Furthermore, preliminary experiments on rats treated with 6-hydroxydopamine to destroy the catecholamine nerve terminal systems (23) have not revealed any obvious loss of immunoreactive boutons (results not shown).

The restriction of postsynaptic immunoreactivity to those junctions in which presynaptic staining is also observed raises the possibility that, whatever transmitters are associated with the existence and function of protein I presynaptically, the same relationship may also extend to the postsynaptic cell. Furthermore, the existence of punctate immunoreactivity observed by light microscopy in the external layer of the median eminence suggests that protein I immunoreactivity may offer an additional guide to possible "secretory" sites even when other methods of microscopic examination fail to reveal classic ul-

trastructural features of axo-axonic junctions (16, 17, 24). Protein I may be a specific component of certain junctions in which events modifiable by  $\text{Ca}^{2+}$ , cyclic nucleotides, or other intracellular regulatory agents occur. Further studies will be required to quantitate these sites and their possible selective association with certain neurotransmitter systems (1, 18, 19), to determine the nature of this protein's function at these sites, and to disclose whether other substrates of protein kinases may also exist at these or other synaptic sites.

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