Proc. Natl. Acad. Sci. USA
Vol. 76, No. 11, pp. 5977–5981, November 1979
Neurobiology

Widespread distribution of protein I in the central and peripheral nervous systems

(protein phosphorylation/phosphoprotein/immunohistochemistry/radioimmunolabeling of gels)

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Contributed by Paul Greengard, August 24, 1979

ABSTRACT **Protein I, a naturally occurring substrate for cyclic AMP-dependent and calcium-dependent protein kinases, previously has been found only in mammalian brain, where it has been demonstrated to be located in neurons. Various tissues and organs outside the brain have now been examined for the possible occurrence of protein I, by using both an immunohistochemical approach and a chemical procedure involving radioimmunolabeling of polyacrylamide gels. Protein I has been found in the inner plexiform layer of the retina, in the posterior pituitary, and in the autonomic nervous system. In tissues composed predominantly of cells other than nerve cells, immunoreactivity was present only where innervation was present. Protein I appeared to be localized in some, but not all, nerve terminals and synapses.**

Proteins Ia and Ib, collectively referred to as protein I because their properties are similar, are endogenous substrates for cyclic AMP (cAMP)-dependent and calcium-dependent protein kinases in mammalian brain (1, 2). An immunohistochemical study of the localization of protein I in brain is reported in the companion article (3). In this article we describe the observations obtained by using an immunohistochemical and an immunochemical approach to examine tissues outside the brain for the possible presence of protein I.

MATERIALS AND METHODS

Materials. Soybean trypsin inhibitor and phenylmethylsulfonyl fluoride were purchased from Sigma; Trasylol was from Mobay Chemical Corp. (New York); ^{125}I-labeled protein A [from *Staphylococcus aureus*; specific activity 30 mCi/mg (1 Ci = 3.7×10^{10} becquerels)] was from Amersham. Purified goat antibodies against rabbit IgG were conjugated to peroxidase (4) by J. Rossier (Roche Institute, Nutley, NJ).

Preparation and Characterization of Anti-Protein I Antiserum. Protein I purified from bovine brain was used to immunize rabbits as described (3). The specificity of the antiserum was verified by three different criteria using extracts and homogenates of whole rat brain: (*i*) in the double agar immunodiffusion test, the antiserum gave a single band of precipitation when tested against a Triton extract; (*ii*) in precipitation experiments on pH 3–pH 6 extracts (1) involving rabbit anti-protein I antiserum followed by goat IgG raised against rabbit IgG, only protein I was specifically precipitated; (*iii*) by radioimmunolabeling of sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gels of total homogenates, only two peptides, with the same electrophoretic mobility as purified protein I, were labeled (Fig. 1).

Radioimmunolabeling of Gels. $NaDodSO_4$/polyacrylamide slab gel electrophoresis of total homogenates of rat brain and of other organs was performed as described (1), except that the separation gel contained 8% polyacrylamide and 0.21% *N,N'*-methylenebisacrylamide. Animals were killed by decapitation, and the organs were quickly removed, weighed, and homogenized in 20 vol (wt/vol) of ice-cold 5 mM Tris·HCl (pH 7.4) containing a mixture of substances to prevent proteolysis [soybean trypsin inhibitor, 0.1 mg/ml; 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Trasylol, 50 units/ml; and 50 μM phenylmethylsulfonyl fluoride]. Within 1 min from the beginning of the homogenization, aliquots of

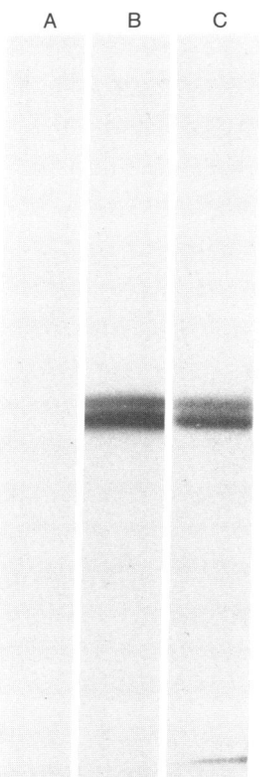

FIG. 1. Autoradiograph of 8% $NaDodSO_4$/polyacrylamide gels showing comigration of immunoreactive material in brain homogenate with authentic protein I and indicating specificity of antiserum against protein I. Rat brain total homogenate was applied to lanes A and B (2 μg per lane) and purified ^{32}P-labeled protein I standard was applied to lane C. After electrophoresis, lanes A and B were cut from the gel and processed for radioimmunolabeling by the antibody/^{125}I-labeled protein A technique. Lane A was incubated in the presence of preimmune serum; lane B was incubated in the presence of antiserum raised against protein I. In the rat brain homogenate, only two peptides, with the same electrophoretic mobilities as proteins Ia and Ib, were labeled by ^{125}I. The identity of the two immunoreactive peptides with proteins Ia and Ib was further suggested by the relative amounts of ^{125}I and ^{32}P label in the upper and lower bands.

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Abbreviations: cAMP, cyclic AMP; $NaDodSO_4$, sodium dodecyl sulfate.

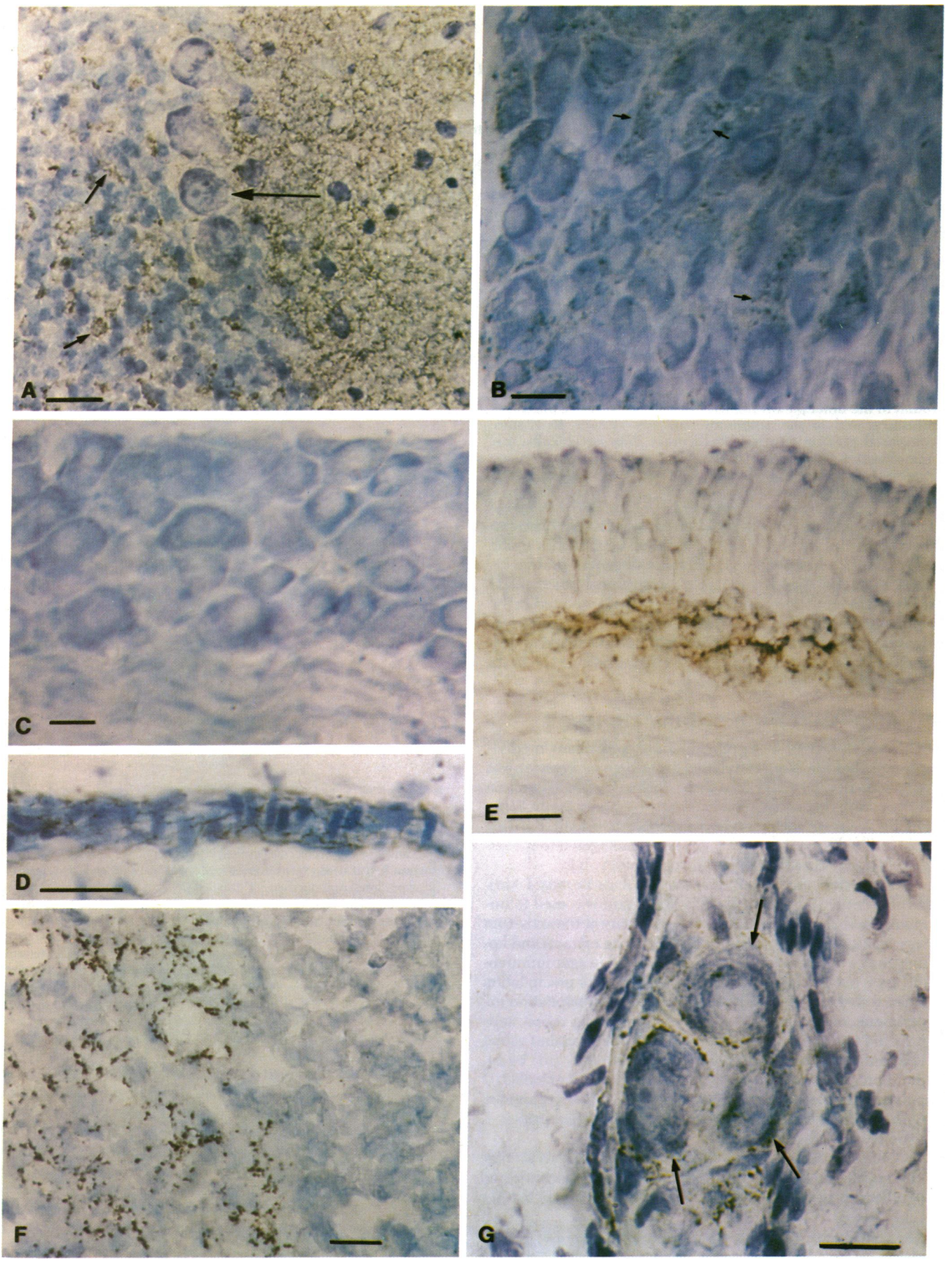

FIG. 2. (*Legend appears at the bottom of the next page.*)

the homogenates to be used for electrophoresis were mixed (2:1, vol/vol) with 6% (wt/vol) $NaDodSO_4$/6% (vol/vol) mercaptoethanol/30% (vol/vol) glycerol, 0.1% bromophenol/0.3 M Tris·HCl, pH 6.8 ($NaDodSO_4$ solution) and immediately boiled for 2 min. Protein concentration was determined on an aliquot of the original homogenate (5), and the ratio of protein to $NaDodSO_4$ in each sample to be loaded was adjusted to 1:15 (wt/wt) by adding an appropriate volume of a 1:3 dilution of the $NaDodSO_4$ solution and then boiling for an additional 1 min. At the end of the electrophoresis, gels were fixed for 3 hr in water/methanol/acetic acid, 46:46:8 (vol/vol), and subsequently processed by the procedure of Adair *et al.* (6) with the following modifications: washing of the gels between steps was carried out for at least 12 hr and in much larger volumes of buffer; incubation of gels in the antiserum (diluted 1:250), or in the same dilution of preimmune serum, was carried out in the cold; ^{125}I-labeled protein A (^{125}I-protein A) incubations were carried out in the minimal volume sufficient to cover the gels; the amount of ^{125}I-protein A used was variable (5×10^4–1×10^6 cpm/ml), with higher concentrations being used for gels in which lower concentrations of protein I were expected.

Preparation of Tissues. Male Sprague–Dawley rats or Syrian hamsters were anesthetized and perfused with fixative as described (7), except that 0.15 M phosphate buffer was used. The various organs were rapidly removed, dissected if necessary into smaller pieces, and further processed as described (7). Immunohistochemical studies were carried out with minor modifications of the procedures described in protocol 2 of ref. 3. Cryostat sections (10–20 μm thick) were incubated overnight at 4°C in the presence of the primary antiserum (diluted 1:250–1:500). Sections were then washed and incubated with peroxidase-conjugated goat IgG raised against rabbit IgG. Both antibody incubation steps were carried out in the presence of 0.3% Triton X-100. In most cases, sections were counterstained with toluidine blue at the end of the peroxidase reaction. In sections treated with preimmune serum or with serum from nonimmunized rabbits, little or no background or nonspecific staining was detected.

RESULTS AND DISCUSSION

Histochemical Demonstration of Protein I Immunoreactivity in Nerve Terminals and Synapses of Several Tissues. Immunohistochemical studies indicated that protein I immunoreactivity was by no means limited to the brain; such immunoreactivity was also found in neuronal elements of many other tissues. Some of the results are illustrated in Figs. 2 and 3. The most abundant immunoreactivity was present in organs and tissues consisting primarily of neural tissue, such as the posterior pituitary, the retina, and several ganglia of the autonomic nervous system. In agreement with results obtained in the brain (3), staining occurred in peripheral tissues only where nerve terminals and synapses are known to be present: the posterior pituitary is composed almost entirely of secretory endings and of nerve terminals forming synaptic contacts with the secretory endings (10, 11); in the retina the staining occurred in the inner plexiform layer which is one of the only two layers containing synapses (the other is the outer plexiform layer); in the sympathetic and parasympathetic ganglia, staining was present around and among neurons, where synapses are located. In contrast, no staining was observed within neuronal cell bodies (nuclear layers of the retina; cell bodies of the ganglionic neurons). Nor was staining observed in axons away from the terminals: no immunoreactivity was found in the nerve fiber layer of the retina or in a number of major and minor nerve trunks examined. In the nodose ganglion, a sensory ganglion devoid of synapses (8), no staining occurred.

In tissues and organs composed predominantly of cells other than nerve cells, immunoreactivity was present only where innervation was present. For instance, no staining was observed in the anterior pituitary, where nerve elements are almost totally absent (11), whereas scattered dots of immunoprecipitate were seen in the intermediate pituitary, in which sparse synapses are present (11). Similarly, in the adrenal gland, staining was present in the medulla, which is highly innervated, but not in the cortex, which receives almost no innervation (10).

The pattern of staining was always consistent with the architectural organization of nerve terminals and synapses in the various tissues as studied by other procedures (e.g., catecholamine-induced fluorescence; immunohistochemical studies at the light microscopic level with antisera against various neurotransmitter molecules). Thus, where nerve terminals and synapses have been described as punctate structures or short filaments, protein I immunoreactivity reproduced this pattern. This was the case, for instance, around the acini of the parotid gland (12), among the parenchymal cells of the intermediate lobe of the pituitary (13), and in the islets of Langerhans (14). In contrast, where nerve terminals have been described as branched, beaded, fibrillar structures (9, 15), an analogous pattern of immunoreactivity was observed with anti-protein I antiserum. This was the case, for example, in the mucosa of the stomach and gut and in all the types of smooth muscle that we examined (the muscle layers of the gut, iris, vas deferens, and blood vessels). In the case of the smaller blood vessels, the fibers of immunoprecipitate formed an anastomotic network around the wall.

FIG. 2 (*on preceding page*). Light microscopy of different tissues tested for anti-protein I immunoreactivity by the indirect immunoperoxidase technique. Immunoreactive material appears brownish. All sections have been counterstained with toluidine blue. (Calibration bars: *A*, *C–G*, 25 μm; *B*, 40 μm.) (*A*) Rat cerebellar cortex, showing granule cell layer at left, Purkinje cell layer (long arrow indicates a Purkinje cell) in center, and molecular layer at right. The peroxidase staining is abundant in areas that contain synapses; it is particularly abundant in the molecular layer and in the region occupied by the glomeruli (short arrows) in the granule cell layer. Note the absence of staining within cell bodies. (*B*) Rat superior cervical ganglion. Dots of immunoreactive material are scattered among cell bodies of sympathetic neurons that are peroxidase negative. Most of the dots (arrows) are at some distance from the perikarya, in agreement with the known abundance of synapses on dendrites at a distance from the perikarya in the superior cervical ganglion (8, 9). (*C*) Rat nodose ganglion. Perikarya of ganglionic neurons (top) and nerve fibers (bottom) are visible in the picture. No immunoreactive material is observed in this ganglion, in agreement with the absence of synapses in sensory ganglia (8). (*D*) Blood vessel in the connective tissue adjacent to the kidney hilus in the rat. The wall of the vessel is surrounded by interconnected beaded filaments of immunoreactive material. (*E*) Rat stomach. A ganglion of the Auerbach plexus enclosed between the longitudinal layer (top) and the circular layer (bottom) of the smooth muscle wall can be seen. Immunoreactive material is abundant in the ganglion and forms a network around ganglionic neurons. Filaments of immunoreactive material, strongly suggestive of varicose nerve terminals, are also visible in the muscular layers. (*F*) Hamster adrenal, showing medulla at left and cortex at right. In the medulla, but not in the cortex, abundant dots of immunoreactive material are present among parenchymal elements which do not show immunoreactivity. (*G*) Rat ciliary ganglion. Three ganglionic cells (arrows) can be seen surrounded by dots of immunoprecipitate which form a pericellular net around the perikarya. Note the different distribution of the peroxidase staining in the superior cervical ganglion (*B*) and in the ciliary ganglion. In the former, synapses are known to be primarily axodendritic (9); in the latter, synapses occur predominantly on the cell bodies or on short dendrites in close proximity to the perikarya (8, 9).

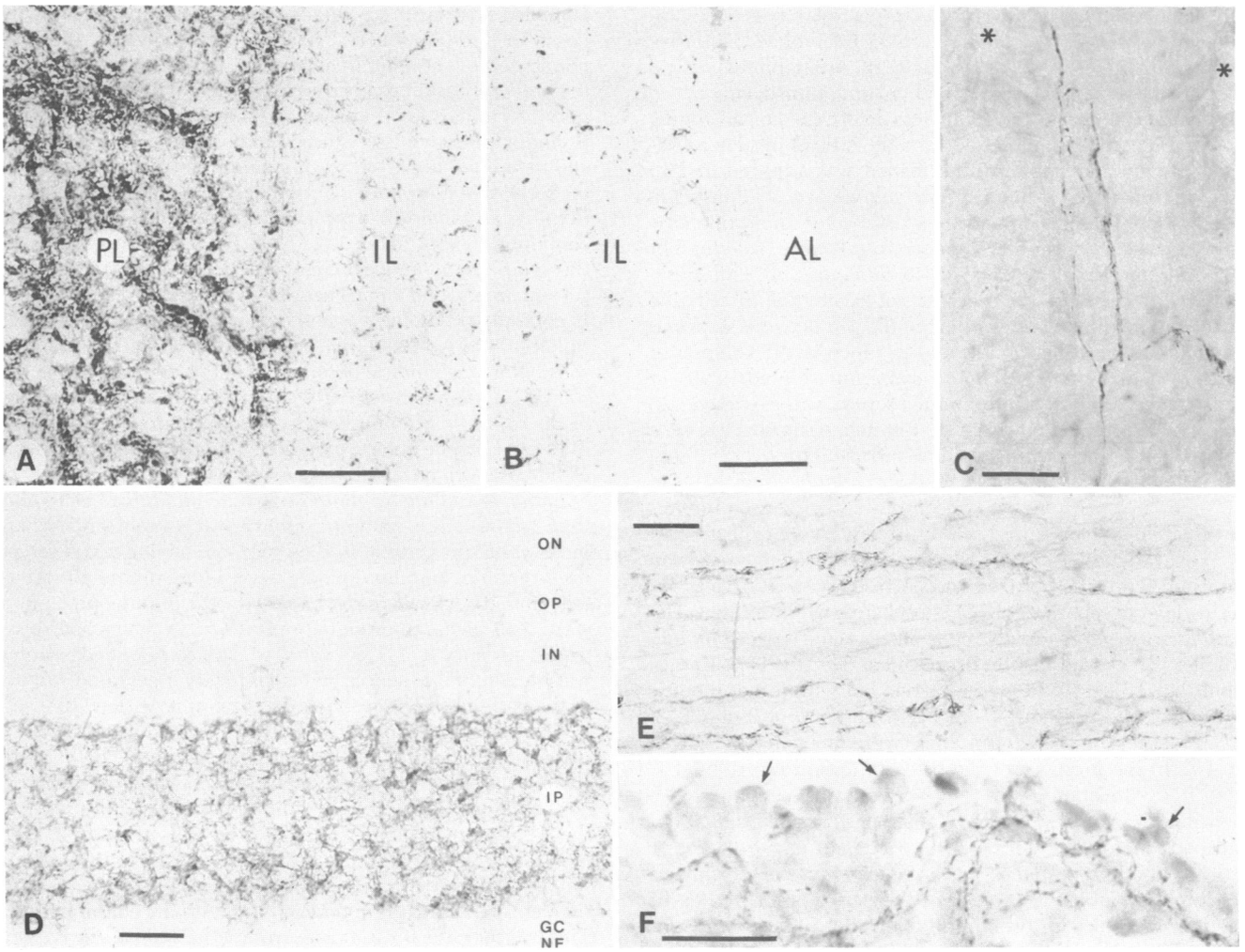

FIG. 3. Light microscopy of different rat tissues tested for anti-protein I immunoreactivity by the indirect immunoperoxidase technique. *A*, *B*, and *D* have not been counterstained; *C*, *E*, and *F* have been slightly counterstained with toluidine blue. (Calibration bars: *A–C*, *E*, *F*, 25 μm; *D*, 50 μm.) (*A* and *B*) Pituitary. PL, posterior lobe; IL, intermediate lobe; AL, anterior lobe. Abundant immunoreactivity is present in the posterior lobe which is mainly composed of neurosecretory terminals and of nerve terminals making synapses onto them; dots of immunoprecipitate are scattered among parenchymal cells of the intermediate lobe which is known to be sparsely innervated; no staining is present in the anterior lobe which receives no innervation. (*C*) Stomach mucosa. A varicose branched fiber of immunoreactive material penetrates the mucosa. Asterisks indicate the lumen of stomach glands. (*D*) Retina. ON, outer nuclear layer; OP, outer plexiform layer; IN, inner nuclear layer; IP, inner plexiform layer; GC, ganglion cell layer; NF, nerve fiber layer. Immunoreactivity is detectable only in the inner plexiform layer, where the immunoprecipitate forms a complex varicose fibrillary network. The slight, nonspecific darkening of the outer plexiform layer was also observed in control sections treated with preimmune serum. (*E*) Circular smooth muscle layer of the rat stomach wall. Varicose interconnected filaments, running parallel to the major axis of the muscle cells, are visible. They are often seen in close proximity to blood vessels. (*F*) Iris. Varicose interconnected filaments of immunoprecipitate are abundant. Cells of the iris epithelium, highly counterstained with toluidine blue, are indicated by the arrows.

Protein I Immunoreactivity Is Present Only in Some Synapses and Terminals. Protein I immunoreactivity did not seem to be characteristic of all terminals and synapses. For instance, of the two layers of the retina that contain synapses, only the inner plexiform layer was stained. No staining was observed at the neuromuscular junction or around the pancreatic acini [which are cholinergically innervated (16)], in contrast to the presence of stain around the parotid acini [abundantly innervated by catecholaminergic fibers (12)]. The absence of immunoreactivity from the motor end plate and from the pancreatic acini suggests that at least some types of cholinergic terminals do not contain protein I. That the absence of protein I is a general characteristic of cholinergic terminals cannot be concluded as yet. Immunoreactivity was present at other sites (such as in the ganglia of the autonomic nervous system and in the adrenal medulla) that are predominantly cholinergically innervated. However, other types of terminals (catecholaminergic or peptidergic) are also present at these sites (9, 17) and these might be responsible for the protein I immunoreactivity.

There was a striking association between catecholaminergic innervation and protein I immunoreactivity. In all tissues examined in which abundant catecholaminergic terminals are present [iris (9), vas deferens (15), parotid gland (12, 16), islets of Langerhans (14), blood vessels, inner plexiform layer of the retina (18), and intermediate and posterior pituitary (13)], protein I immunoreactivity was present. In addition, the pattern of staining in those locations closely resembled the pattern of catecholamine-induced fluorescence. However, there is increasing evidence for a more complex innervation of these tissues. In particular, there appears to be a widespread distribution of peptidergic fibers at all these sites (19, 20).

Immunochemical Evidence for the Occurrence of Protein I Outside the Brain. It was important to determine that the results of the immunohistochemical studies were attributable to protein I and not to antigenic sites on other peptides that

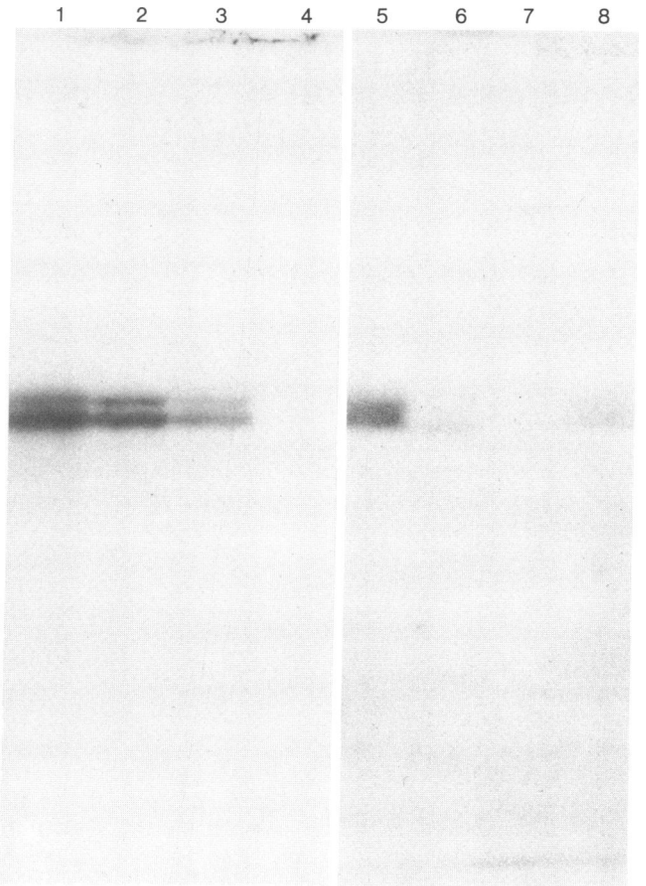

FIG. 4. Autoradiographs showing radioimmunolabeling of Na-DodSO$_4$/polyacrylamide gels by the antibody/^{125}I-protein A technique. An aliquot (200 μg of protein) of rat tissue homogenate was applied to each lane. Lanes: 1, cerebrum; 2, cerebellum; 3, posterior pituitary; 4, anterior pituitary; 5, vas deferens; 6, stomach; 7, pancreas; 8, parotid. In order to increase the sensitivity of the method for some of the tissues (lanes 5–8), a higher concentration of ^{125}I-protein A and a longer time of exposure of the x-ray film to the gel were used. In all tissues except the anterior pituitary, a doublet with the same electrophoretic mobility as protein I was labeled by ^{125}I. The anterior pituitary was negative even when processed by the conditions used for lanes 5–8.

might have crossreacted with our antiserum. For this purpose, homogenates of several organs that we had examined by immunohistochemistry were subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis and then the gels were processed by a radioimmunolabeling technique (6). This technique allows the identification, in polyacrylamide gels, of peptide bands containing the antigenic sites against which an antiserum is directed. Preliminary experiments with brain tissue had indicated that this procedure is able to detect extremely small amounts of protein I and that it provides a semiquantitative estimation of the amount of protein I present in the gel. In all tissues in which immunoreactivity was detected histochemically, a doublet specifically reacting with the antiserum against protein I (but not with preimmune or nonimmune serum) and with the same electrophoretic mobility as protein I could be demonstrated (Figs. 1 and 4). No other bands reacting with the antiserum were observed. Those tissues that showed the greatest amount of protein I immunoreactivity in the morphological experiments also showed the most intense staining in the autoradiographs (e.g., cerebellum $\gg$ vas deferens $>$ pancreas $>$ anterior pituitary).

Other Comments. The localization of protein I in terminal endings of axons, where storage and release of neurosecretory material occur, suggests that a function of this protein may be to mediate or modulate processes related to storage or release of neurotransmitters. The absence of protein I from some nerve terminals and from secretory cells other than neurons indicates that protein I does not play a universal role in vesicular secretion. On the other hand, the widespread distribution of protein I both in the brain (3) and in neural structures outside the brain (present study) strongly suggests that the role of protein I in neural tissue is not limited to a single neurotransmitter system. An important future step toward the clarification of the function of protein I will be the identification of the types of terminals in which it is present. The occurrence of protein I in various locations outside the brain will permit morphological as well as physiological experiments in systems in which the synaptic organization is much less complicated than in the brain.

This work was supported by U.S. Public Health Service Grants DA-01627, MH-17387, and NS-08440 (to P.G.) and MH-29466 and AA-03504 (to F.E.B.) and by a grant from the McKnight Foundation (to P.G.). This work was done during the tenure of a Muscular Dystrophy Association Research Fellowship awarded to P.D.C.

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