Widespread distribution of protein I in the central and peripheral nervous systems
(protein phosphorylation/phosphoprotein/immunohistochemistry/radioimmunolabeling of gels)

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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 immunochromic 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); 125I-labeled protein A [from Staphylococcus aureus; specific activity 30 mCi/mg (1 Ci = 3.7 × 1010 becquerels)] was from Amersham. Purified goat antibodies against rabbit IgG were conjugated to peroxidase (4) by J. Rossier (Roche Institute, Nutley, N.J).

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 antiprotein I antiserum followed by goat IgG raised against rabbit IgG, only protein I was specifically precipitated; (iii) by radioimmunolabeling of sodium dodecyl sulfate (NaDodSO4)/polyacrylamide gels of total homogenates, only two peptides, with the same electrophoretic mobility as purified protein I, were labeled (Fig. 1).

Radioimmunolabeling of Gels. NaDodSO4/polyacrylamide slab gel electrophoresis of total homogenates of rat brain and of other organs was performed as described (1), except that the

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Abbreviations: cAMP, cyclic AMP; NaDodSO4, 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) NaDodSO4/6% (vol/vol) mercaptoethanol/50% (vol/vol) glycerol, 0.1% bromphenol/0.3 M Tris-HCl, pH 6.8 (NaDodSO4 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 NaDodSO4 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 NaDodSO4 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; 125I-labeled protein A (125I-protein A) incubations were carried out in the minimal volume sufficient to cover the gels; the amount of 125I-protein A used was variable (5 × 103–1 × 105 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 (5), staining occurred in perineural cells 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 the 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 the types of stomach plexuses 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.
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).

Immunohistochemical 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
might have crossreacted with our antiserum. For this purpose, homogenates of several organs that we had examined by immunohistochemistry were subjected to NaDodSO₄/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 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 > 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.

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