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Cellular and subcellular localization of protein I in the peripheral nervous system

(phosphoproteins/neurotransmitter vesicles/chromaffin granules)

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ABSTRACT **The cellular and subcellular distribution of protein I, a major brain phosphoprotein, has been studied in the peripheral nervous system. The levels of protein I in various peripheral nerves and innervated peripheral tissues were determined by radioimmunoassay and radioimmunolabeling of polyacrylamide gels. The results indicated that protein I is present throughout the peripheral nervous system. Denervation studies of adrenal medulla and iris suggested that the protein I contained in peripheral tissues is localized to the neuronal elements innervating those tissues. Protein I was found to be enriched in neurotransmitter vesicle fractions of peripheral nervous tissue. Moreover, protein I appeared to be transported from cell bodies to axon terminals at least partly in association with neurotransmitter vesicles.**

Protein I is a neuron-specific protein (1) that is a prominent endogenous substrate in brain for both cyclic AMP-dependent (1) and calcium/calmodulin-dependent (2–4) protein kinases. The state of phosphorylation of protein I has been shown to be regulated by impulse conduction (5), neurotransmitters (6, 7), and depolarizing agents (6–8) in intact preparations of neuronal tissue. The distribution of protein I in the central nervous system has been studied by light and electron immunocytochemistry (9, 10), subcellular fractionation (11, ‡), and radioimmunoassay (12). Protein I is present throughout the central nervous system. It is concentrated in presynaptic nerve terminals, where it is associated at least partially with neurotransmitter vesicles. Protein I has also been detected immunocytochemically, apparently in axon terminals, throughout the peripheral nervous system (13).

In the present study we have determined the cellular and subcellular distribution of protein I in various peripheral nervous tissues which have the advantage over brain of being better defined, less complex, and more easily manipulated experimentally.

MATERIALS AND METHODS

Materials. Pure protein I was kindly supplied by Louis J. DeGennaro of our laboratory. ^{125}I-Labeled protein A (specific activity, 30 mCi/mg; 1 Ci = 3.7×10^{10} becquerels) was purchased from Amersham and protein A-bearing *Staphylococcus aureus* cells (SAC) (Pansorbin) were from Calbiochem-Behring.

Denervation. Rats with denervated adrenal glands (achieved by transsection of the splanchnic nerve), as well as sham-operated control rats, were obtained from Zivic-Miller Laboratories (Allison Park, PA). Individual adrenal glands were analyzed 2–4 weeks after surgery, at which time denervation is complete (14). Irides were sympathetically denervated by superior cervical ganglionectomy of Sprague–Dawley rats from Anticimex (Stockholm, Sweden). This procedure causes complete degeneration of iris sympathetic nerves within 48 hr (15). Sixteen normal irides and 30 denervated irides, taken 5 days after surgery, were pooled for protein I analysis.

Subcellular Fractionation. All of the sucrose solutions used in the fractionation procedures were buffered with 5 mM potassium phosphate (pH 7.3). Adrenal medulla was fractionated according to the following modification of the procedure of Helle and Serck-Hanssen (16). The medullae from 10 bovine adrenals or 20 rat adrenals were dissected in the cold and homogenized with an Ultra-Turrax for 90 sec in 5 vol of ice-cold 0.25 M sucrose. The homogenate was centrifuged at 800 × *g* for 10 min and the supernatant (S1) was centrifuged at 10,000 × *g* for 30 min, yielding a supernatant (S2) and a pellet (P2). The pellet was resuspended in 13 ml of ice-cold 0.25 M sucrose and layered on a discontinuous sucrose gradient. The gradient consisted of a 0.6 M sucrose upper part and either a 1.6 M sucrose lower part (bovine adrenals) or a 1.2 M sucrose lower part (rat adrenals). The gradient was centrifuged at 145,000 × *g* for 60 min. The material that collected at the top of the gradient (0.25 M) and at the 0.25/0.6 M and 0.6/1.6 M (or 0.6/1.2 M) interfaces was collected as fractions 1, 2, and 3, respectively. The material that pelleted through the bottom step was collected as fraction 4 and represents the chromaffin granule fraction. Fractions 1–3 were diluted to isotonicity with ice-cold water. Fractions 1–3 and S2 were then centrifuged at 145,000 × *g* for 60 min. The resulting pellets and fraction 4 were stored frozen prior to analysis for protein I.

Large noradrenergic vesicles were isolated from splenic nerve axons according to the following modification of the procedure of Klein *et al.* (17). Splenic nerve axons from 10–15 bovine spleens were dissected in the cold, minced with scissors, and homogenized with an Ultra-Turrax for 2 min in ice-cold 0.25 M sucrose. The homogenate was centrifuged at 10,500 × *g* for 20 min and the supernatant was layered on a sucrose density gradient. The gradient consisted of an upper part of 0.4 M sucrose in H_2O below which was a continuous gradient from 0.6 M sucrose in $H_2O/^2H_2O$ (1:1) to 1.2 M sucrose in ^2H_2O. The gradient was centrifuged at 280,000 × *g* for 90 min and then divided into eight fractions. Fractions 1–7 were successive portions of the continuous gradient starting from the top, and fraction 8 was the gradient pellet. Fractions 1–7 were diluted to isotonicity with ice-cold water and centrifuged at 145,000 × *g* for 60 min. The resulting pellets were resuspended in water; aliquots were taken for analysis of norepinephrine content and

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Abbreviation: DBH, dopamine β-hydroxylase.

‡ Huttner, W. B., De Camilli, P., Schiebler, W. & Greengard, P., *Abstracts of the 11th Annual Meeting of the Society for Neuroscience*, Los Angeles, CA, Oct. 18–23, 1981, p. 441 (abstr.).

the remaining material was lyophilized and stored frozen prior to analysis for protein I. Fraction 8 was resuspended in 2 ml of ice-cold water and stored frozen prior to analysis for protein I.

Small noradrenergic vesicle fractions were prepared from vas deferens of normal or castrated male rats according to the procedure of Fried *et al.* (18, 19). Crude small noradrenergic vesicle fractions were also prepared from rat heart and rat salivary gland by the same procedure.

Radioimmunoassay of Protein I. The protein I content of peripheral nervous tissues or fractions of the tissues was determined by a detergent-based radioimmunoassay (12). The tissue samples or fractions to be assayed were homogenized in 1% $NaDodSO_4$ (approximately 4 mg of protein per ml) and boiled for 5 min. The boiled $NaDodSO_4$ homogenates were then centrifuged in a Beckman Microfuge for 2 min. The supernatants ("$NaDodSO_4$ extracts"), which contained essentially all of the protein I, were either assayed immediately or frozen and stored for up to 1 week before being assayed. At least four concentrations of each extract were assayed in triplicate. Each of the $NaDodSO_4$ extracts analyzed competed in the assay in a manner parallel to that of protein I purified from rat or bovine brain, indicating the immunological similarity of the protein I from the different sources. The total protein content of the $NaDodSO_4$ extracts was determined according to the procedure of Lowry *et al.* (20) with bovine serum albumin as standard.

Radioimmunolabeling of Protein I in Polyacrylamide Gels. The protein I content of $NaDodSO_4$ extracts was also estimated by using a modification (21) of a procedure (13, 22) for radioimmunolabeling protein I in polyacrylamide gels.

Determination of Norepinephrine Levels. Norepinephrine was measured by a high-pressure liquid chromatography method using electrochemical detection (23). α-Methyldopamine was used as internal standard. Standard curves were linear in the range used.

Axoplasmic Transport of Protein I. Bovine spleens were removed and splenic nerve segments 4–6 cm long were dissected at room temperature within 45 min after death. A loose ligature was placed in the middle of each nerve segment and the nerve was then crushed by constricting the ligature against a metal rod for 30 sec. The nerve segments were incubated in oxygenated Tyrode's solution (137 mM NaCl/2.7 mM KCl/12 mM $NaHCO_3$/1.8 mM $CaCl_2$/0.5 mM $MgCl_2$/0.4 mM NaH_2PO_4/0.01% ascorbic acid/1% glucose, pH 7.4) for 30 min at room temperature and then for 4 hr at 37°C. After incubation, nerve segments underwent immersion fixation in freshly depolymerized 4% paraformaldehyde in phosphate buffer (pH 7.4) and were processed for immunocytochemistry with the secondary antibody labeled with rhodamine (to be described elsewhere).

The left sciatic nerve of several rats was ligated, as described above, *in situ* while the animals were under chloral hydrate anesthesia. Rats were perfused with 250 ml of ice-cold freshly depolymerized 4% paraformaldehyde in phosphate buffer (pH 7.4) 24 hr later. The presence of protein I in ligated and in contralateral control nerves was examined by immunocytochemistry with the secondary antibody labeled with fluorescein isothiocyanate.

RESULTS

Protein I Levels in Intact Peripheral Nervous Tissues. Protein I levels in some peripheral nerves and innervated peripheral tissues, as determined by radioimmunoassay, are shown in Table 1. Protein I levels determined by radioimmunoassay were in excellent agreement with those determined by radioimmunolabeling of gels, within the experimental error of the gel technique (data not shown). The level of protein I in the superior cervical ganglion was approximately 2% of that in cerebral cortex but was severalfold higher than that in the other peripheral tissues studied. The much greater amount of protein I in cortex than in peripheral tissues is consistent with the much greater density of synapses in the cortex as well as with staining patterns of protein I (ref. 13; unpublished data).

Table 1. Protein I levels in the peripheral nervous system determined by radioimmunoassay

Tissue	Protein I, fmol/mg protein
Superior cervical ganglion (rabbit)	1,040
Splenic nerve (cow)	490
Adrenal medulla (cow)	275
Vas deferens (rat)	255
Iris (rat)	220
Adrenal medulla (rat)	140
Cerebral cortex (rat)	54,000

Data represent the means of determinations on tissues from at least four animals. Protein I levels in rabbit, cow, and human cerebral cortex were similar to those in rat cerebral cortex (data not shown). Protein I levels in rat superior cervical ganglion were similar to those in rabbit ganglion.

Effect of Denervation. Light immunocytochemical studies suggested that the protein I contained in peripheral tissues, such as adrenal medulla and iris, is present in the neuronal elements innervating those tissues (ref. 13; unpublished data; see also Fig. 4 *d* and *e*). The possible neuronal localization of protein I was further studied by determining the effect of denervation on the protein I content of adrenal medulla and iris. Transsection of the splanchnic nerve destroys the preganglionic cholinergic nerve terminals that innervate the adrenal medulla. Extracts of denervated adrenal medulla contained no detectable protein I when assayed either by radioimmunoassay or by radioimmunolabeling of gels (data not shown). In order to enhance the sensitivity of detection and thereby better define an upper limit for the amount of protein I that remains after denervation, the protein I contained in normal and denervated adrenal medulla extracts was concentrated by immunoprecipitation and then analyzed by radioimmunolabeling of gels as described (7). In contrast to the protein I detected in immunoprecipitates of normal adrenal medulla extract (Fig. 1 *Left*; lane 1), no protein I was detected in immunoprecipitates of denervated gland extract (lane 2). Protein I was readily detected, however, in immunoprecipitates of denervated gland extract that contained 5% (vol/vol) of normal gland extract (lane 3). Therefore, denervated adrenal medulla contained less than 5% of the amount of protein I present in intact adrenal medulla.

Fig. 1 *Right* shows the protein I content of normal (lane 4) and denervated (lane 5) iris. Sectioning of the adrenergic fibers to the iris, which left the cholinergic and sensory fibers intact, resulted in a 45% decrease in the protein I content of the iris as determined by radioimmunoassay and radioimmunolabeling of gels.

Subcellular Localization of Protein I in Peripheral Nervous Tissue. Protein I in brain is at least partially associated with neurotransmitter vesicles (9, 11, ‡), and it was of interest to determine whether protein I in peripheral nervous tissue has a similar distribution. The bovine splenic nerve and rat vas deferens represent the best available sources, among mammalian peripheral tissues, for the isolation of highly purified and well-characterized neurotransmitter vesicles (19). Large dense-cored vesicles containing norepinephrine can be obtained, with 80–90% purity, from bovine splenic nerve axons (17). Similarly, small dense-cored vesicles containing norepinephrine can be

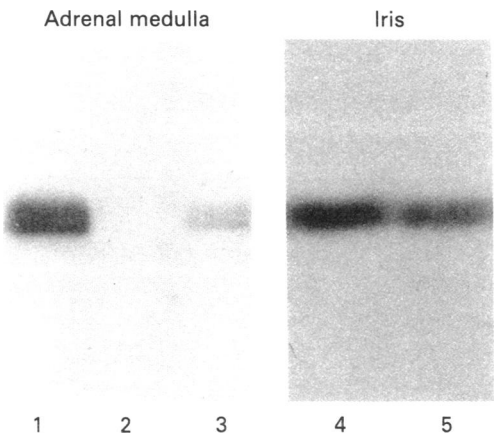

FIG. 1. Autoradiograph of gels illustrating the effect of denervation on the protein I content of adrenal medulla and iris. (*Left*) Protein I immunoprecipitates were prepared from intact adrenal medulla extract (lane 1), denervated adrenal medulla extract (lane 2), and denervated adrenal medulla extract containing 5% (vol/vol) intact adrenal medulla extract (lane 3). In each case, the extracts that were subjected to immunoprecipitation contained 1 mg of protein. The immunoprecipitates were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis and the gels were then radioimmunolabeled for protein I. (*Right*) Extracts (containing 200 μg of protein) prepared from intact (lane 4) and adrenergically denervated (lane 5) iris were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis and the gels were then radioimmunolabeled for protein I. The protein I content of intact and denervated iris, determined by radioimmunoassay, was 220 and 120 fmol of protein I per mg of protein, respectively.

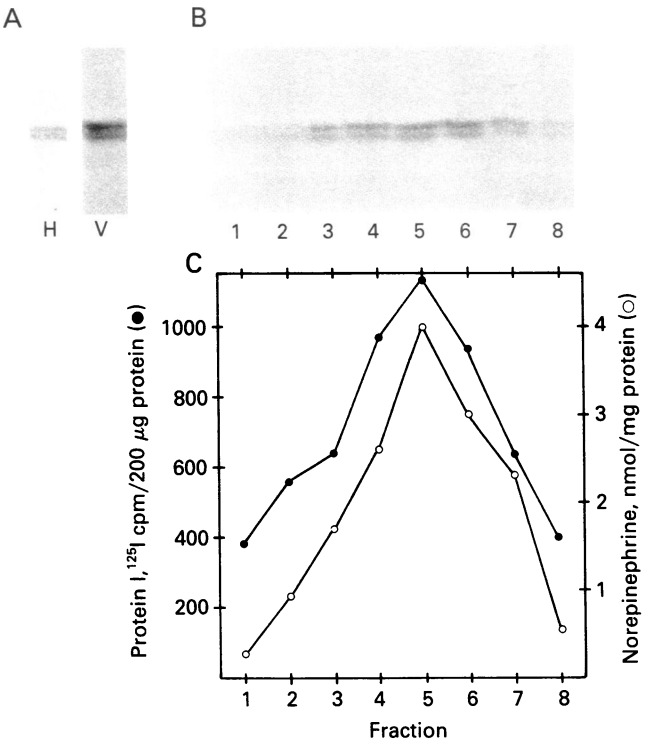

FIG. 2. Protein I and norepinephrine content of fractions of bovine splenic nerve. (*A*) Extracts (containing 200 μg of protein) prepared from whole splenic nerve homogenate (H) and from the large dense-cored vesicle fraction of splenic nerve (V) were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis and the gel was then radioimmunolabeled for protein I. An autoradiograph of the gel is shown. The protein I levels in the homogenate and vesicle fractions, determined by radioimmunoassay, were 490 and 3,900 fmol of protein I per mg of protein, respectively. (*B*) Extracts (containing 200 μg of protein) prepared from sucrose gradient fractions of splenic nerve were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis and the gel was then radioimmunolabeled for protein I. An autoradiograph of the gel is shown. (*C*) Protein I (●) and norepinephrine (○) content of sucrose gradient fractions. Protein I values were obtained from the gel shown in *B*.

obtained with 40–60% purity from the vas deferens of castrated rats (18, 19). These large and small noradrenergic vesicles are qualitatively similar in many respects to the catecholamine-containing chromaffin granules of the adrenal medulla (19), and the latter were included in this study for comparative purposes. Protein I levels in homogenates of splenic nerve, vas deferens, and adrenal medulla were compared to protein I levels in vesicle fractions of these tissues by radioimmunolabeling of gels. The level of protein I in splenic nerve vesicle fractions was approximately 8-fold higher than that in whole splenic nerve homogenate (Fig. 2*A*). Protein I was also increased in the vesicle fraction of vas deferens where it was approximately 2-fold higher than in whole vas deferens homogenate (data not shown). In contrast to splenic nerve and vas deferens, chromaffin granules contained less protein I than did whole adrenal medulla homogenate. This was the case for bovine adrenal gland (Fig. 3, lanes 1 and 5) as well as for rat adrenal gland (data not shown).

To investigate further the subcellular distribution of protein I in bovine splenic nerve, a more detailed fractionation was performed (Fig. 2). Fig. 2*B* is an autoradiograph of a radioimmunolabeled gel that shows the distribution of protein I in sucrose gradient fractions of splenic nerve. Fig. 2*C* compares the amounts of protein I and norepinephrine in each fraction. The distribution of protein I and norepinephrine closely paralleled each other, indicating that protein I is at least partially associated with norepinephrine storage particles in splenic nerve axons.

Axoplasmic Transport of Protein I. The presence of protein I in splenic nerve axons (Fig. 2) suggests that protein I is transported along axons. Further evidence for the axoplasmic transport of protein I, as well as for its localization at nerve endings, is shown in Fig. 4. Protein I was found to accumulate mainly proximal, but also distal, to the ligature in both bovine splenic nerve (4.5 hr after ligation) (Fig. 4*A*) and rat sciatic nerve (24 hr after ligation) (Fig. 4*C*). Norepinephrine, another substance that undergoes axoplasmic transport, also accumulates mainly proximal to ligations (24). Fig. 4*B* shows the accumulation of dopamine β-hydroxylase (DBH), a protein associated with neurotransmitter vesicles (see ref. 25), both proximal and distal to the ligation of splenic nerve.

Subcellular Localization of Protein I in Adrenal Medulla and Other Innervated Organs. The lower concentration of protein I in isolated chromaffin granules than in adrenal medulla homogenates (Fig. 3, lanes 1 and 5) suggests that these granules are not the main subcellular storage site for protein I in this tissue. Furthermore, the >95% decrease in protein I levels of the adrenal medulla observed after denervation suggests that the localization of protein I in the adrenal medulla is largely, and possibly exclusively, presynaptic. The distribution of protein I in subcellular fractions of bovine (Fig. 3) and rat (data not shown) adrenal medulla was consistent with these observations. Thus, protein I was increased in the sedimentable part of the microsomal supernatant (S2, lane 2) and in the top fraction of the sucrose gradient (fraction 1, 0.25 M, lane 3) compared to whole homogenate (lane 1). These fractions are known to contain small neurotransmitter vesicles derived from the presynaptic elements of the tissue (19, 26, 27). In addition, the 0.6/1.6 M sucrose interface (fraction 3, lane 4), which appears to be of heterogeneous composition, also contained detectable levels of protein I.

The distribution of protein I in subcellular fractions of other

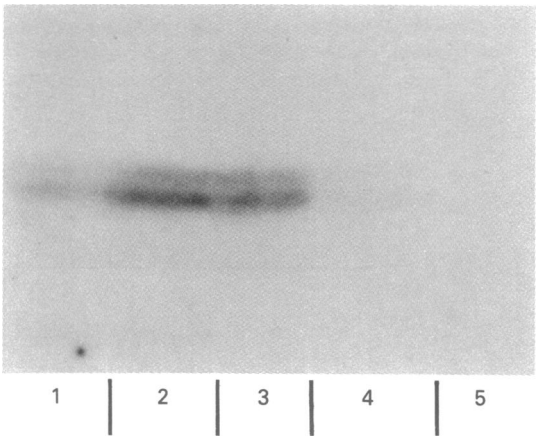

FIG. 3. Autoradiograph of a gel radioimmunolabeled for protein I, illustrating the distribution of protein I in various fractions of bovine adrenal medulla. Extracts (containing 200 μg of protein) were prepared from whole homogenate (lane 1), from the microsomal supernatant (S2; lane 2), from the top fraction of the sucrose gradient (fraction 1; lane 3), from the 0.6/1.6 M sucrose interface (fraction 3; lane 4), and from the chromaffin granule fraction (fraction 4; lane 5) and subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis and the gel was then radioimmunolabeled for protein I.

organs with a peripheral innervation, such as heart and salivary gland, was also investigated. These organs yield crude neurotransmitter vesicle preparations when fractionated according to the procedure used for rat vas deferens (see ref. 19). The level of protein I in these tissues was very low (<100 fmol of protein I per mg of protein). However, most of the immunoreactivity detected by radioimmunolabeling of gels was found in those gradient fractions (0.4–0.6 M sucrose) known to contain small neurotransmitter vesicles (data not shown).

DISCUSSION

Protein I has been shown, by cytochemical procedures, to be present throughout the central and peripheral nervous systems (9, 10, 13, ‡). In the present study, we have quantitated the levels of protein I in various peripheral nerves and innervated peripheral organs. Decreases in protein I levels, observed in response to the denervation of the adrenal medulla and iris, support immunocytochemical observations (ref. 13; unpublished data; see also Fig. 4 *d* and *e*) indicating that protein I is localized to the neuronal elements of peripheral tissues. Protein I is not present in non-neuronal tissues, including even tissues developmentally related to neurons, such as adrenal chromaffin cells. The enrichment of protein I in neurotransmitter vesicle fractions prepared from noradrenergic nerves and the virtual absence of protein I in chromaffin granules of the adrenal medulla contrast dramatically with the general biochemical similarity (19) of these two types of organelle.

The loss of protein I following the transsection of either cholinergic nerves (adrenal medulla denervation) or adrenergic nerves (iris denervation) suggests that protein I is present in neurons of both types. This finding is consistent with cytochemical observations (unpublished data) and with the observation that the level of protein I in cultured rat sympathetic neurons is the same whether the neurons have a cholinergic or an adrenergic phenotype (unpublished data). The presence of protein I in neurons containing different neurotransmitter systems in peripheral nervous tissues is consistent with observations in brain that protein I is present in most, and possibly in all, presynaptic nerve terminals (ref. 10; unpublished data).

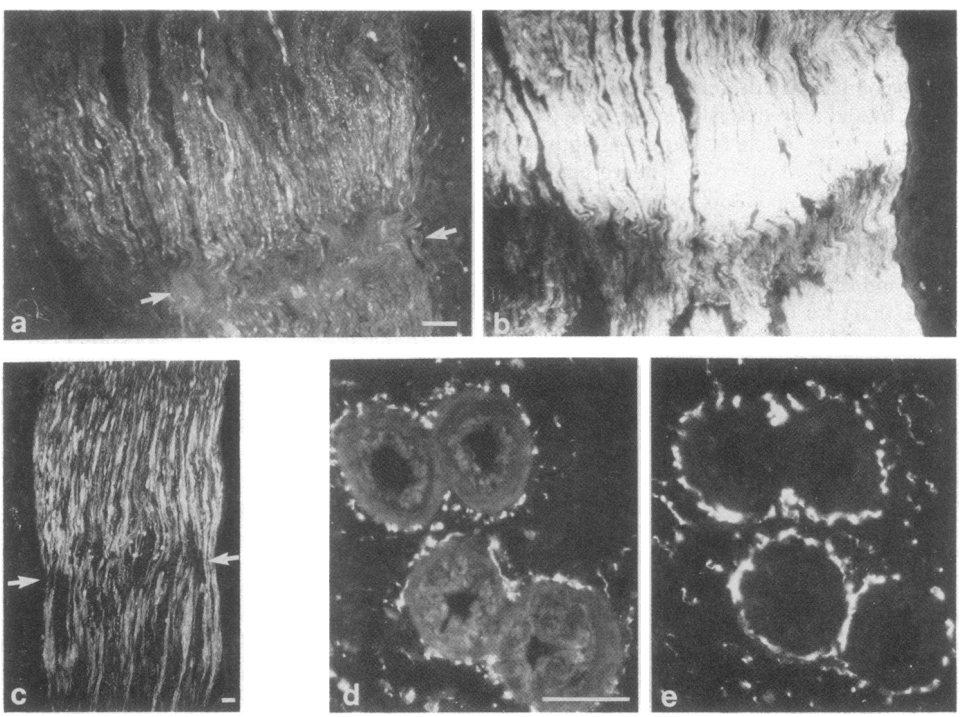

FIG. 4. Immunocytochemical localization of protein I and dopamine β-hydroxylase (DBH) in nerves and blood vessels. Bovine splenic nerve axons show the accumulation of protein I mainly proximal (up) to the ligation (arrows) (*A*) and show the accumulation, in an adjacent section, of DBH both proximal and distal to the ligation (*B*). Rat sciatic nerve (*C*) shows the accumulation of protein I mainly proximal (up) to the ligation (arrows). Protein I was not detected in contralateral control nerves (not shown). Bovine intrasplenic blood vessels show a similar distribution, in adjacent sections, of protein I (*D*) and of DBH (*E*) around the vessels. Because of differences in photographic exposure time, characteristics of primary antisera, nature of fluorescent label, etc., the brightness of the immunoreactive products does not provide a measure of the relative amounts of antigen present in the five sections shown. (Calibration bars, 50 μm.)

The concentration of protein I at synapses led to the suggestion (12) that the measurement of protein I may provide a procedure for determining the density of nerve terminals in neuronal tissue. Such an approach could be of considerable value because a detailed morphometric examination of the regional distribution of nerve terminals in the peripheral and central nervous systems, a difficult and tedious undertaking, has not been carried out. The validity of using protein I levels as an indicator of synaptic density is supported by the present study which shows that peripheral nervous tissues, which contain many fewer synapses than brain, also contain much less protein I. The ratio of the level of protein I in the superior cervical ganglion to that in cerebral cortex is approximately 1:50 (Table 1). We have calculated a similar value (approximately 1:50 to 1:100) for the ratio of the density of synaptic endings, determined morphometrically, in ganglion (8.8×10^6 synapses per mm^3) (28) to that in cortex ($5–10 \times 10^8$ synapses per mm^3) (29). Moreover, the autonomic ground plexus of the iris consists of roughly equal amounts of adrenergic and cholinergic nerves (30). In addition, there is a sparse distribution of sensory nerves. Removal of the superior cervical ganglion therefore should remove roughly half of the total amount of presynaptic nerve terminals in the iris, which agrees well with the observed loss of 45% of the protein I content.

Protein I in brain appears to be at least partially associated with neurotransmitter vesicles (9, 11, ‡). Results obtained in the present study indicate that protein I in the peripheral nervous system is also at least partially associated with neurotransmitter vesicles. Furthermore, the enrichment of protein I in vesicle fractions of splenic nerve axons suggests that the transport of protein I from cell bodies to axon terminals may occur in association with neurotransmitter vesicles. The association of protein I with neurotransmitter vesicles, but not with secretory granules of non-nervous tissues such as adrenal chromaffin granules, suggests that protein I may play a role in a neuron-specific regulation of the release process rather than in the release process *per se*.

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