Histol Histopath (1986) 1: 203-212

Histology and Histopathology

Substance P-like immunoreactivity in rat and cat carotid bodies: Light and electron microscopic studies

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Summary. Substance P-immunoreactive (SP-I) structures in the carotid bodies of rats and cats were examined with the light and electron microscopes. In both species SP-I varicose nerve fibers were located singly in the interstitial connective tissue in close association with blood vessels. They were small unmyelinated fibers enveloped in a common Schwann cell sheath with other SP-negative fibers. Some of SP-I fibers contained large dense-cored granules and small clear vesicles in addition to microtubules and mitochondria and probably represented nerve fiber varicosities. The latter often were found incompletely invested by Schwann cell sheaths. SP-fibers were found occasionally in the envelopes of supporting cells at the periphery of parenchymal cell groups. However, none of the nerve terminals making synaptic contacts with glomus cells exhibited SP-like immunoreactivity. In cat carotid bodies some glomus cells showed moderate to intense SP-like immunoreactivity.The intense SP-I glomus cells displayed numerous dense-cored vesicles of 85 to 140 nm in diameter and frequently showed synaptic contacts with SP-negative nerve terminals. In rat carotid bodies we were unable to detect consistent SP-immunoreactivity in glomus cells. Our results do not favor the hypothesis that SP is a neurotransmitter/modulator in the chemoreceptor afferents synapsing on glomus cells in either the cat or rat carotid body. However our results support the hypothesis that SP in cat glomus cells may play a role in the modulation of chemoreceptor activity.

Key words: Carotid body - Substance P-EM immunocytochemistry - Rat and cat

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Introduction

It is generally accepted that primary chemoreceptor afferents synapse peripherally on glomus cells in the carotid body. An accumulation of data suggests that substance P (SP) is a possible candidate for the neurotransmitter/ neuroinodulator in primary baro-and chemoreceptor afferent neurons. These data include: (1) immunocytochemical localization of SP in certain neuronal perikarya in the petrosal and nodose ganglia, in some nerve fibers of the sinus and aortic nerves (e.g. Lundberg et al., 1978; Gamse et al., 1979; Helke et al., 1980a,b; Jacobowitz and Helke, 1980; Katz and Karten, 1980; Helke, 1982) and, in some axon terminals in the nucleus of the tractus solitarius (NTS) (e.g., Pickel et al., 1979; Voorn and Buijs, 1983; Kalia et al., 1984; Maley, 1985); (2) modulatory effects of SP following application of the neuropeptide into the region of the NTS where central processes of the 9th and 10th cranial afferents terminate (e.g. Haeusler and Osterwalder, 1980; Hedner et al., 1981; Henry and Sessle, 1985), and (3) depletion of SP in the NTS following denervation of the 9th and 10th cranial nerves (e.g. Gillis et al., 1980; Helke et al., 1980a). However, none of these data provide direct evidence that SP is present in the primary chemo-and baroreceptor afferent neurons. Several investigators have demonstrated the presence of a few SP immunoreactive nerve fibers in the carotid body (Lundberg et al., 1979; Jacobowitz and Helke, 1980; Cuello and McQueen, 1980; Chen et al., 1984) but synaptic connections between SP-fibers and glomus cells which are supposed to be essential for chemoreception (Verna et al., 1975; Zapata et al., 1977; Smith and Mills, 1979; Eyzaguirre and Fidone, 1980; Nishi et al., 1981; Monti-Bloch et al., 1983 a,b) have not been identified. On the other hand, the functional roles of SP in the chemoreflex are still controversial (Reis et al., 1981; Mueller et al., 1982; Furness et al., 1982; Lorez et

al., 1983; Carter and Lightman, 1983, 1985). The present research was undertaken in order to visualize SP-immunoreactive (SP-1) structures in the carotid bodies of rats and cats at the ultrastructural level.

Materials and methods

Fourteen adult rats (Sprague-Dawley) and 10 adult cats which included both sexes were anesthetized with sodium pentobarbital (50 mg/kg body weight) and were perfused through the left ventricle with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.2-7.3) for 30 min. a1 room temperature. Carotid bodies were excised and fixed an additional 3 to 16 hr. in the perfusate at 4°C before being transferred to a cold phosphate buffer containing 8% sucrose (for I hr.) and 30% sucrose (overnight). The [issues were then frozen with acetone which had been precooled with dry ice, and sectioned in a cryostat at 40 µm or hand-sliced following 2 cycles of freezing and thawing. Both frozen and hand-sliced sections were preincubated in 10% normal goat serum (NGS) in phosphate buffered saline (PBS) with or without 0.1% Triton X-100 for I hr. at 25°C and then were immunocytochemically processed for SP using peroxidase-antiperoxidase (PAP, Sternberger et al., 1970) or avidin-biotin-peroxidase complex (ABC) (Hsu et al., 1981). The rabbit anti-SP serum (lmmunonuclear Corp., Stillwater, Minnesota) was diluted to 1:2000 (for PAP technique) or 1:5000 (for ABC technique) times with 1% NGS in PBS (staining times, 16 hr. at 25°C and 60 hr. at 4°C). For control staining either the normal rabbit serum or anti-SP serum preabsorbed with synthetic SP (100 µg/ml of original anti-SP serum; Sigma Chem. Co., St. Louis, Missouri) were used in place of the anti-SP serum at the same dilutions. Following diaminobenzidine (DAB)-H_2O_2 incubation some of the frozen sections were mounted on microscopic slides with a glycerine-PBS mixture and photographed. Other tissue slices were postosmicated and processed for electron microscopy. Five to 10 consecutive thin sections were individually picked up with 50 mesh copper grids. Sections either uncontrasted with a heavy metal (unless otherwise mentioned) or contrasted with lead citrate were examined in a JEOL 100B or a JEOL 100CX electron microscope.

Results

In 40µm frozen sections treated with the anti-SP serum, most varicose nerve fibers (varicosities interconnected by thin segments) showing SP-like immunoreactivity occurred singly in the interstitial connective tissue in both rat and cat carotid bodies (Figs. 1, 4). Most of these SP-like immunoreactive (SP-I) fibers were closely associated with blood vessels. Occasionally, however some appeared to skirt around groups of parenchymal cells. The number of SP-1 fibers was much smaller than DBH-I ones in the carotid body (Chen et al., 1985). In cat carotid bodies some parenchymal cells also showed moderate to strong SP-like immunoreactivity. The number of stained parenchymal cells varied from specimen to specimen. In a few instances, a majority of parenchymal cells exhibited staining (compare Figs. 4 and 5). Intensely SP-I cells occurred singly or in small groups and often were provided with long, single processes (Fig. 4). In rat carotid bodies, we were unable to detect consistent SP-like immunoreactivity in parenchymal cells.

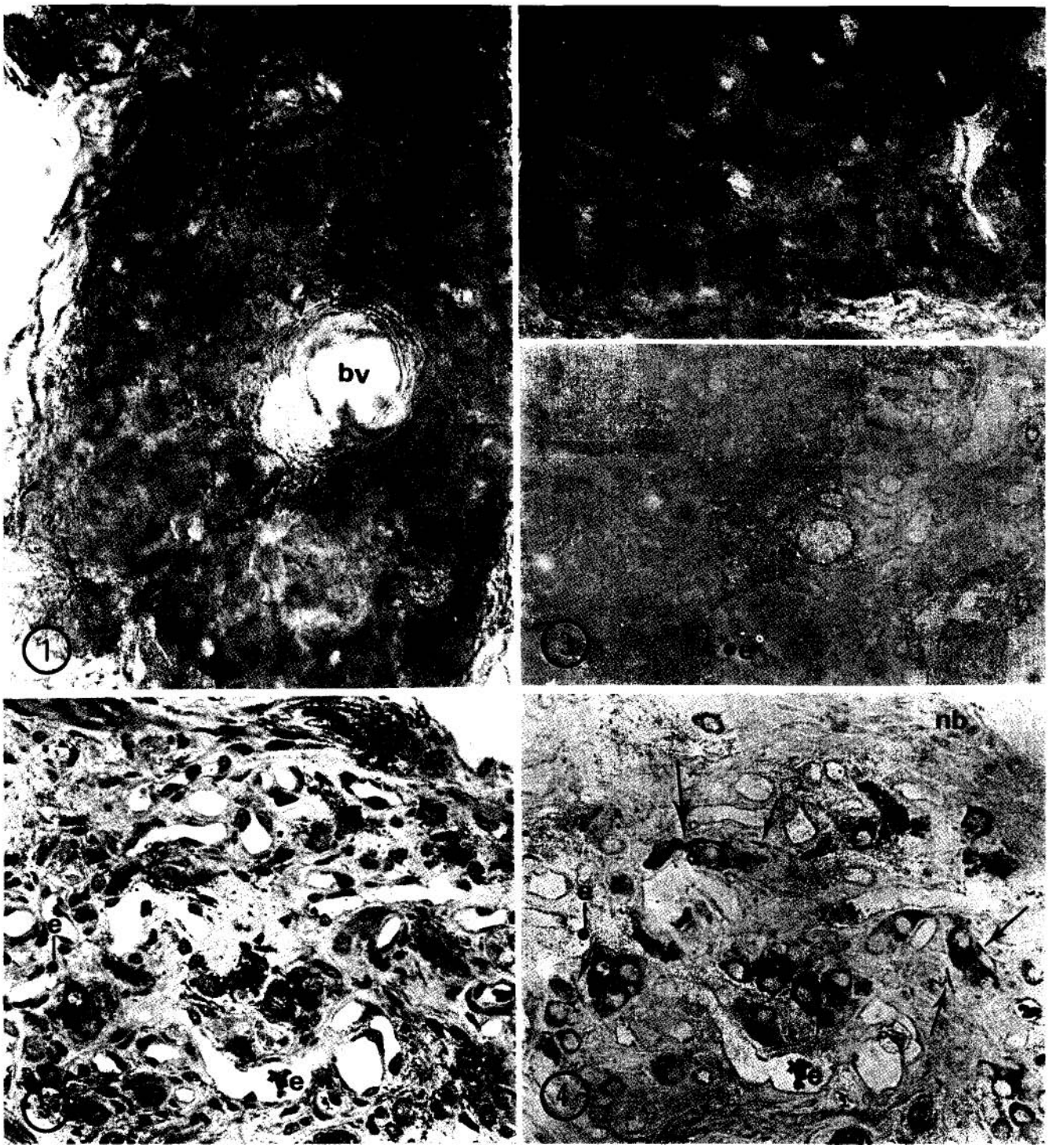
Figs. **1** and 2. Light micrographs from 40 µm frozen sections of a rat carotid body treated with anti-SP (Fig. 1) and normal rabbit sera (Fig. 2). Parenchymal cell islands (examples are shown by surrounding arrowheads) are barely visible in these micrographs. Not all SP-I fibers (arrows) are in the focal plane. However a few are seen in a whole cross section of the carotid body in Fig. 1. bv, blood vessel. x 440

Figs. 3, **4** and **5.** Photomicrographs from 2 µm Epon sections of a cat carotid body. Tissue slices had been treated with normal rabbit (Fig. *3)* and anti-SP (Figs. 4, 5). In Fig. 4 parenchymal cells exhibit various extents of SP-like immunoreactivity. Small arrows point to SP-I fibers and a large arrow to a long single process of an intensely stained cell. In Fig. 5 (the same section as in Fig. 4 but after staining with toluidine blue) counterstaining has reduced the contrast ot DAB reaction product but has brought up many spindle-shaped nuclei, most of which are unlikely glomus cells. nb, nerve fiber bundle; *e,* erythrocyte. x 440

Figs. **6** and **7.** Electron micrographs from a rat carotid body showing SP-I fibers (SP) in the vicinity of a parenchymal cell group (Fig. 6) and in enclosure of a supporting cell process (Fig. 7, lead citrate stained). A few vesicles in glomus cells appear to show some reaction product in dense-cored vesicles (arrows). In Fig. 7 a nerve terminal (nt) making synaptic contact is unlabeled. ly, lysosome. x 25,000

Figs. **8** and **9.** Micrographs from a cat carotid body showing SP-I fibers associated with a parenchymal cell group (Fig. 8, lead citrate stained) and a blood vessel (Fig. 9). In Fig. 8 one of the 2 SP-I fibers (SP) appears unaccompanied by unlabeled fibers and contains small clear vesicles (small arrows) in addition to dense cored vesicles (large arrows). Some dense-cored vesicles in the glomus cell display weak SP-like imrnunoreactivity. In Fig. 9, one of the two profiles of SP-I fibers displays many small clear vesicles and appears to be largely uncovered by the Schwann sheath. x 26,000

Fig. **10.** Low magnification electron micrograph of a cat carotid body. The thin section had been cut roughly perpendicular to the surface (lower right corner of the micrograph showing some non-specific staining) of a tissue slice. Among 6 glomus cells with nuclear profiles (numbered), SP-I granules can be seen in Nos. 1 through 3 at this magnification. At a higher magnification (see Fig. 11) other glomus cells (Nos. 4 and 5) also show weak to moderate staining in some dense cored vesicles. mf, myelinated fiber; s, supporting cell. x 1,900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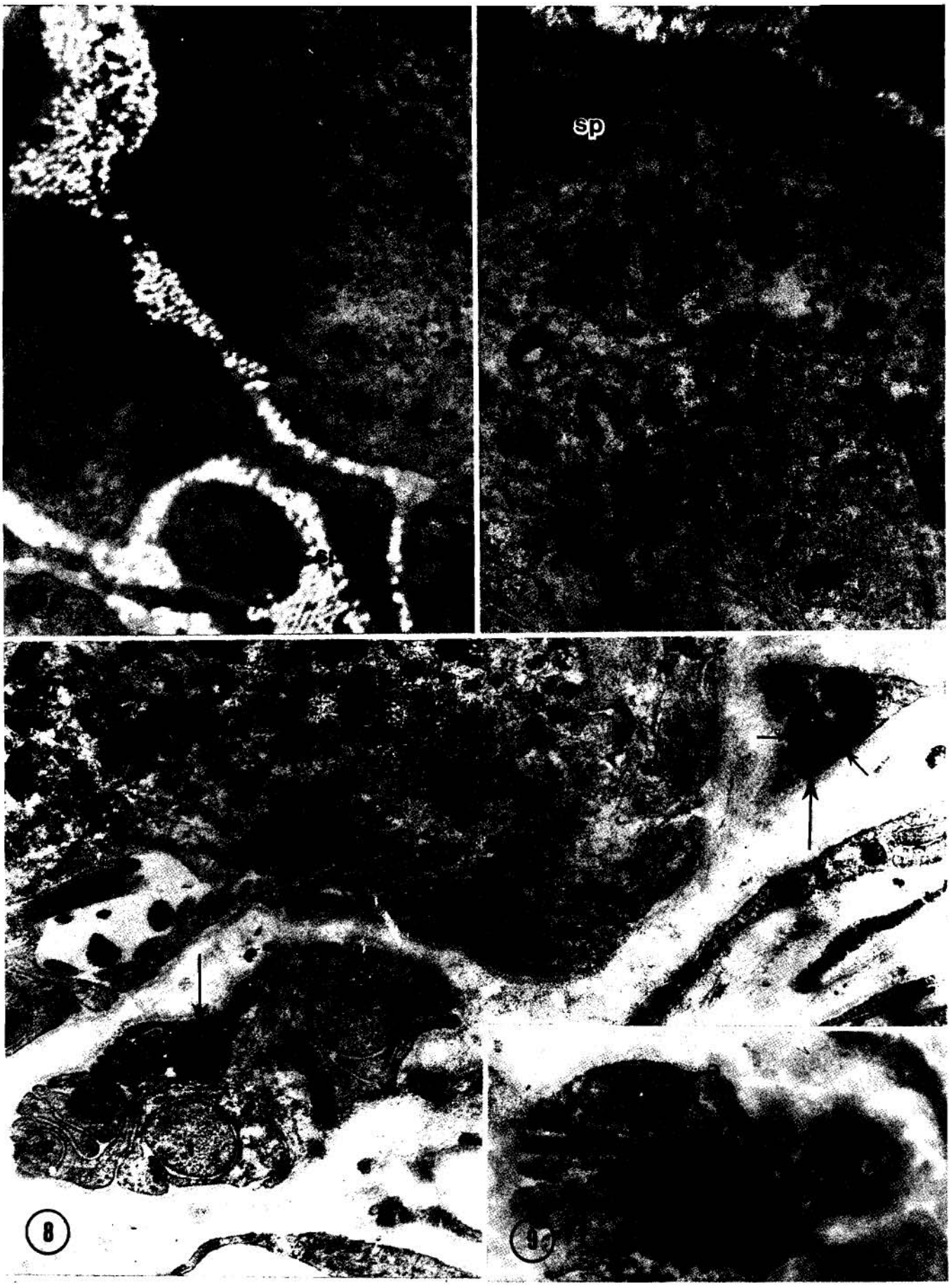
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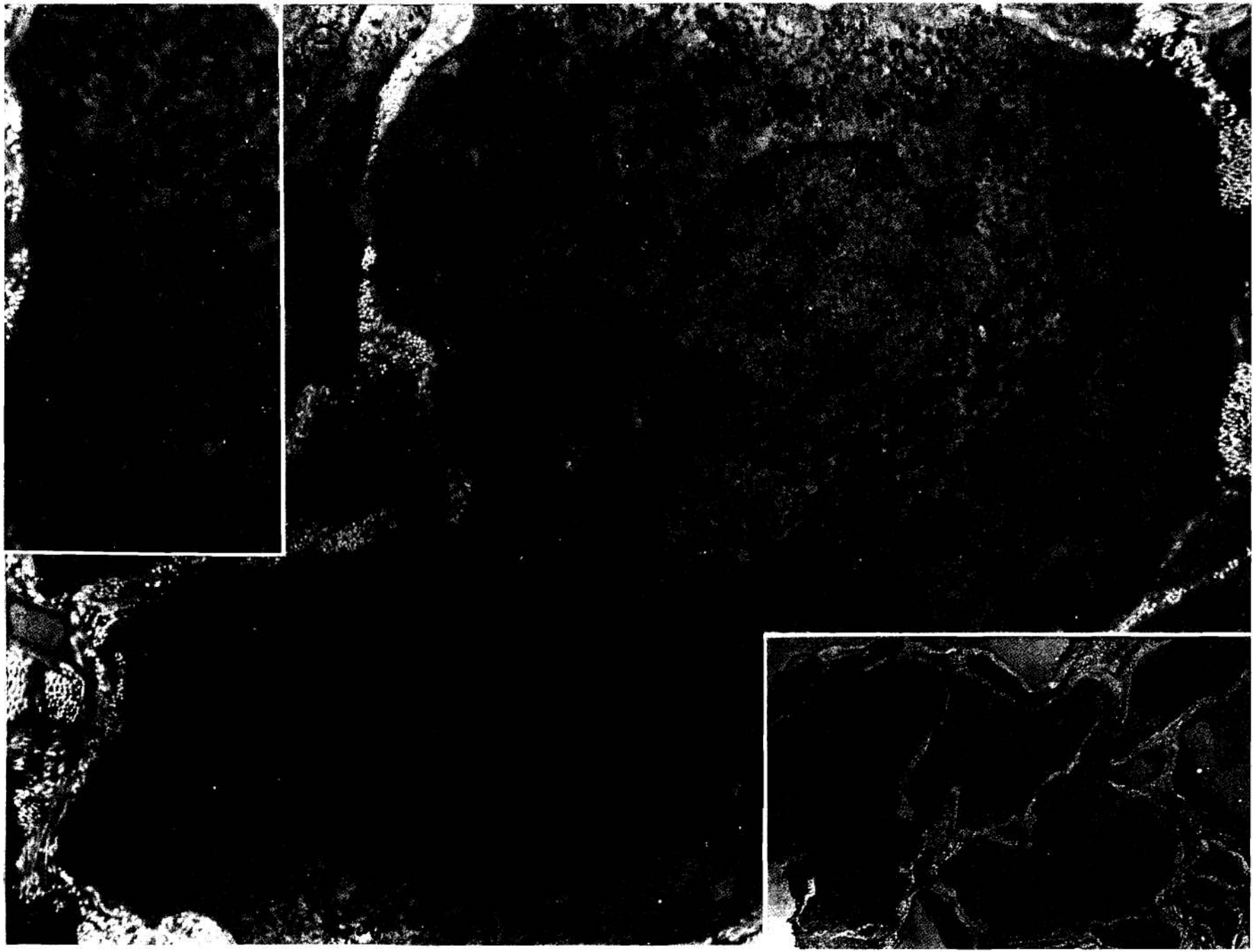

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Fig. 11. Enlargement of parts of glomus cells Nos. 2 through 4 and supporting cell(s) in Fig. 10. In glomus cell No. 4 many dense-cored vesicles located in the cytoplasm to the left of the nucleus show weak to moderate imrnunostaining. sp, SP-fiber; nt, nerve terminal. x 12,000

Fig. 12. Enlargement of a part in Fig. 10 bounded by the rectangle. The glornus cells on the right display granules with weak to moderate immunostaining, while the one on the left (with larger granules) shows no staining in the granules. x 12,000

Fig. 13. Part of an intensely stained SP-I glomus cell and its process apposed by two profiles of nerve terminals (nt). No detectable irnmunoreactivity is seen in these terminals (cat). x 26,000

Fig. 14. An active zone of synapse between an SP-I glornus cell and a nerve terminal (nt) (cat). x 45,000

Fig. 15. A nerve terminal (nt) sliced open prior to immunocytochemical staining. Note that the terminal synapses with an SP-I glomus cell and is unlabeled except at its surface (asterisk) where non-specific staining is seen. x 22,000

In control sections treated with the normal rabbit serum the following structures showed non-specific peroxidative activity: some blood cells, cytoplasmic inclusions, presumably lysosomes, and mast cells (only following ABC techniques; Bussolati and Gugliotta, 1983). Patches of reaction product sometimes were observed on the surfaces of tissue slices (Fig. 10, 15). Preabsorption of the antiserum with SP abolished staining of varicose nerve fibers, but very faint staining of small numbers of glomus cells, seemed to remain. Such faint residual staining was similar to that reported in SIF cells by Dail and Dziurzynski (1985).

Electron microscopy revealed that SP-I fibers were small unmyelinated ones. One or two profiles of SP-I fibers, if present, versus several SP-negative fibers in a common Schwann sheath was the most frequent arrangement. However, more than two profiles of SP-I fibers in a common Schwann sheath were observed occasionally, especially in large nerves. Solitary SP-I fibers (Fig. 8) werc rarely encountered. In a few instances, in both rat and cat carotid bodies, a myelinated fiber was seen accompanied by a group of unmyelinated ones containing an SP-I axon within a common perineurial sheath. The diameter of SP-I fibers ranged from 0.1 to 0.65 µm in rats and from 0.2 to 0.77 µm in cats, therefore some were beyond or barely at the limit of resolution of the light microscope. Small profiles of SP-I fibers contained mitochondria, microtubules and dense-cored vesicles of 85-140 nm in diameter. Additionally, large profiles of SP-I fibers often exhibited small clear synaptic vesicles (Figs. 6-9). The latter profiles probably represented varicose parts of SP-I fibers seen in light microscopy and often were incompletely surrounded by a Schwann sheath. The frequency of occurrence of dense-cored vesicles in SP-I fibers appeared higher in the cat than in the rat carotid bodies. None of the nerve terminals making synaptic contacts with glomus cells exhibited SP-like immunoreactivity (Figs. 7, 11, 13-15), although a few SP-I fibers were observed to be ensheathed in processes of supporting cells at the periphery of parenchymal cell groups in both rat (Fig. 7) and cat (Fig. 1i) carotid bodies.

In cat carotid bodies the parenchymal cells which exhibited SP-like immunoreactivity were glomus cells (Figs. 10, 11). The intensity of staining of glomus cells varied from absent to intense. In general those glomus cells showing intense staining were provided with numerous large dense-cored vesicles of around 140 nm in diameter (Figs. 11, 13) and long processes containing similar vesicles (Fig. 13). Immunoreactivity primarily resided in the dense-cores of vesicles and also varied from undetectable to strong even in the same glomus cells (Figs. 11-15). Most stained vesicles were spherical in contour, but oval or rod-shaped ones were not infrequent. No SP-like immunoreactivity was detected in glomus cells which contained many densecored vesicles whose diameter was larger than 200 nm (Fig. 12). SP-I glomus cells werc often seen to make synaptic contact with nerve terminals containing numerous small clear vesicles. Accumulations of the latter and/or SP-I vesicles on the glomus cell side at active zones of synapses were noted often (Figs. 13-15). In only two of 14 rats examined, a small number of dense-coreci vesicles in a feu. glonius cells appeared to display very faint immunostaining (Figs. 6, 7).

Discussion

Although there has been accumulating data suggesting that SP is a neuromodulator in chemo- and baroreflex primary afferent neurons, there is no direct evidence indicating the presence of SP in these neurons. In the present electron microscopic study we confirmed the presence of small unmyelinated SP-I nerve fibers in both rat and cat carotid bodies (Lundberg et al., 1979; Jacobowitz and Helke, 1980; Cuello and McQueen, 1980; Chen et al., 1984). The majority of these SP-I fibers. including their varicosities covered only partially by Schwann sheaths, appeared in the interstitial connective tissue without making synaptic contact with any structures. Myelinated and large unmyelinated nerve fibers including those making synaptic contact with glomus cells which are supposed to be essential for chemoreception did not display any SP-like immunoreactivity. Accessibility of immunocytochemical reagents to the latter structures did not seem to be a factor for the negative results. Because nerve terminals sliced open .prior to the imrnunocytochemical procedure showed negative staining except at the surface areas where non-specific adhesion of immunocytochemical agents tends to occur (Fig. 15).

In view of the data that neuropeptides synthesized in the perikarya of sensory ganglia are transported to both central and peripheral terminals (Gamse et al., 1979; Brimijoin et al., 1980; Hayashi et al., 1983), our data did not provide positive support for the idea of a neurotransmitter or neuromodulator role of SP in

chernoreceptor primary neurons. However we do not completely rule out the possibility that the amount of SP in these afferents is below the sensitivity for detection in the techniques employed or, that SP synthesized in the perikarya is mainly transported toward the CNS. The latter possibility seems to be unlikely since in the vagus nerve the bulk of SP in the sensory fibers appears to be transported away from the cell bodies towards the periphery rather than towards the CNS (Brimijoin et al., 1980; Keen et al., 1982). In this connection it is noteworthy that in the carotid sinus wall of guinea pigs, SP-like immunoreactivity seems to reside in efferent perivascular plexuses but not in large presumptive baroreceptor terminals (Gorgas et al., 1983) in which neurotransmitter roles of SP have been proposed (Helke et al., 1980a; Gillis et al., 1980). Additionally, the density of SP-nerve fibers in the carotid sinus is no greater than that of the adjacent internal carotid and less than that in the external carotid arteries (Furness et al., 1982). Our unpublished data also indicate similar results in the rat. In addition neonatal administration of capsaicin, a dose resulting in 60% decrease in the SP content of the NTS, has neither affected blood pressure nor baroreflex function (Lorez et al., 1983) and shows no detectable ultrastructural alterations in nerve terminals making synaptic contact with glomus cells in rats (Hansen, unpublished).

The origin and function of small unmyelinated SP-I fibers in the carotid body are not clear at the present time. Using combined retrograde transport of macromolecules and SP immunocytochemistry, Hess (1981) concluded that SP-I fibers in the sinus nerve arise from a few aberrant wandering ganglion cells in the glossopharyngeal nerve. In the aortic nerve, SP-I fibers have been traced back to some neuronal perikarya in the nodose ganglion (Helke et al., 1980b). However, the latter nerve in WKY strain rats probably does not carry chemoreceptor fibers from the aortic body (Sapru et al., 1981). Another source of nerve supply to the carotid body is the superior cervical sympathetic ganglion. SP-like immunoreactivity is rarely localized in the cell bodies of principal ganglion cells in the autonomic ganglia or nuclei following certain manipulations (Kessler et al., 1981, 1983; Maley and Elde, 1981: Kessler and Black, 1982; Black et al., 1982; Dail and Dziurzynski, 1985). However, a source of SP-I fibers via the glomerular branch of the superior cervical sympathetic ganglion to the carotid body does not seem likely, since ganglionectomy does not result in a significant reduction of the number of SP-I fibers in rat carotid bodies (Hess, 1981).

In the taste buds of circumvallate papillae, a chemoreceptor organ innervated by the glossopharyngeal nerve (Nisimoto et al., 1982), the absence of synaptic contact between SP-I fibers and gustatory cells casts doubt about a primary role of SP-I fibers in conductance of sensory information. Rather trophic influences of SP-I terminals to the target organ have been suggested (Yarnasaki et al., 1984). In the vascular wall antidromic intluences of SP-I fibers have been hypothesized (Furness et al., 1982; Matsuyama et al., 1985). In our study SP-I fibers also exhibit small clear synaptic vesicles in their varicosities. They may be encased in processes of supporting cells at the periphery of a parenchymal cell group, but synaptic contact with glomus cells was not observed. In view of the paucity of SP-I fibers as well as all nerve terminals synapsing on glomus cells being SP-negative in the carotid body as is the case in the taste bud (Yamasaki et al., 1984). we believe that the small unmyelinated SP-1 fibers in the carotid body play a very minor role, if any, in the conductance of chemosensory impulse5 toward the CNS.

The present electron microscope study confirms that ail SP-I parenchymal cells are glomus cells in the cat carotid body. It is not clear if differences in SP-like immunoreactivity represent different rypes of glomus cells or the same type under different physiciogicai conditions. Morita et al. (1969) have differentiated four types of glomus sells in :he cat carotid body on the basis of the size of dense-cored vesicles. Although there are considerable differences in the size of vesicles, possibly largely attributable to different fixation and tissue processing, the intensely SP-I glornus ceils in our material fit into type II category or major glornus cells of the latter investigators. The dense-cored vesicles in these glomus cells also display intense DBH imrnunoreactivity (Chen et al., 1984, 1985). Co-exisience of SP and catecholamines in SIF cells has been demonstrated (Schultzberg, 1983; Dail and Dziurzynski. 1985) and the latter share manv common ultrastructural characteristics with glomus cells. Frequent occurrence of synaptic contacts between SP-1 glomus cells and nerve terminals as well as accumulation of SP-I vesicles at active sites of synapses support neuromodulator roles of SP in chemoreceptor transduction in the cat glomus cells (McQueen, 1980; Prabhakar et al., 1984). Failure of consistent localization of SP-I in rat glomus cells in which the cell types and the mode of innervation are more thoroughly understood (McDonald and Mitchell, 1975; Chen and Yates, 1984) than in cats, casts some doubt about the neuromodulator role of SP in the carotid body in general. Maybe the SP content of the glomus cells under normal conditions is barely at or below the sensitivity of the immunocytochemical techniques employed. Experiments which might induce changes in the neuromodulator content of glomus cells, such as hypoxia, hyperoxia or colchicine treatrnent, followed by SP-immunocytochemistry, may shed some light on a neuromodulator role of SP in the rat carotid body.

Acknowledgements. This work was supported in part by the Lousiana Heart Association and American Heart Association Grant No. 83-733 to J.T.H. Dr. Hansen is the recipient of NIH Research Career Development Award.

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Accepted March 10, 1986