Immunocytochemical identification of substance P cells and their processes in rat sensory ganglia and their terminals in the spinal cord: Light microscopic studies

(peroxidase antiperoxidase/immunofluorescence/rhizotomy/transmitters)

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ABSTRACT The indirect immunofluorescence method and the unlabeled primary antibody peroxidase antiperoxidase method are used to demonstrate the substance P (SP) plexus in the spinal cord and SP cells in the sensory ganglia of the rat. The normal untreated and the control side of the dorsal rhizotomized rats show vast SP immunoreactive plexuses in the substantia gelatinosa, central gray, and ventral gray regions of the spinal cord. In each sensory ganglion, approximately 250 SP immunoreactive cells are found singly or in small groups of 2 or 3, near blood capillaries or among ganglion and satellite cells. They contain intensely immunoreactive cytoplasmic granules 0.1–3.0 μm accros. Occasionally, free intensely immunoreactive granules are found in the surrounding tissue near an SP cell but not clearly within the confines of the cell. Another type of immunoreaction has been observed with both methods. A less intense, homogeneous reactivity has been found in lamellae insinuated between ganglion cells and near blood capillaries close to an SP cell; the characteristic disposition of this homogeneous reactivity suggests an extracellular location. Unilateral rhizotomy results in an increased number of immunoreactive SP cells and nerve fibers as well as a more extensive homogeneous immunoreactivity. These results add to existing evidence that SP cells in sensory ganglia send fibers via the dorsal roots to the spinal cord. SP cells, fibers, and terminals do not take up exogenously applied 125I-labeled [Tyr3]SP and cannot be demonstrated by subsequent autoradiography. No neurotensin immunoreactive cells were found in sensory ganglia.

The hypotensive peptide substance P (SP), originally detected from equine brain and gut tissues by Von Euler and Gaddum (1) and characterized by Chang et al. (2) as an undecapeptide, occurs in numerous locations throughout the central nervous system of mammals (refs 3 and 4; V. Chan-Palay, unpublished data). The present report is concerned only with SP distribution in the sensory ganglia and spinal cord of the rat.

Earlier studies indicated that SP is present in the spinal cord and segmental nerve rootlets, with concentrations greater in the dorsal than in the ventral roots. The distribution suggested that it was localized in the primary sensory afferents (5–7). These observations led to the proposal that SP may be a sensory transmitter for the dorsal roots (7). Recent elegant electrophysiological and pharmacological experiments by Japanese investigators (8–14) show that SP is an excitatory transmitter released from primary afferent nerve terminals in the mammalian and frog spinal cord. This effect has been seen also in the cat (15). The release appears to be calcium-dependent and can be evoked by stimulation of the dorsal roots (16). Other experiments in the supraspinal nervous system such as in the hypothalamus (17) and the cuneate nucleus (18) have confirmed excitation of neurons by SP; however, inhibitory actions of SP in the vertebrate central nervous system have also been reported (19, 20). Nevertheless, the evidence strongly suggests that SP is a transmitter substance either in the intact state or as a precursor to smaller transmitter molecules containing the COOH-terminal sequence (14).

Hökfelt et al. (21–23), using the indirect Coons immunofluorescence method, have demonstrated SP immunoreactivity in cells of the spinal and trigeminal ganglia and in nerve fibers of the spinal cord (particularly in the substantia gelatinosa) and many peripheral tissues of the rat and cat such as in the skin of the paws and around sweat glands. Takahashi and Otsuka (13) have demonstrated biochemically that the high concentrations of SP in the dorsal horn of the cat spinal cord are altered after unilateral ligation or sectioning of the dorsal roots. It was found that the SP levels in the dorsal horn fell, whereas levels in the ganglion rose. Hökfelt et al. (22) confirmed these findings in histological preparations with the indirect immunofluorescence technique. In some species such as the cat, SP cells are difficult to demonstrate. Although no specific SP fluorescence was found in the spinal ganglia, dorsal roots, or certain large peripheral nerve trunks in the normal untreated cat, experimental manipulation, such as the application of colchicine directly to the ganglion or compression of the dorsal roots, revealed a number of SP fluorescent neurons. All SP cells described were small, and the most strongly fluorescent material was found in what was described as the peripheral part of the cytoplasm. These experimental manipulations also provoked an increased number of SP fluorescent fibers in the nerve roots proximal to the ganglion and a decreased number of fibers in the dorsal horn of the spinal cord 10 days after rhizotomy.

Thus, evidence from a number of approaches, biochemical and morphological, suggests that immunoreactive fibers in the spinal cord arise from cells in the spinal ganglia. The present study investigates the precise distribution of the immunoreactivity of SP cells in the dorsal root ganglia in normal rats and after dorsal rhizotomy, by means of the indirect fluorescence method and the unlabeled primary antibody, peroxidase antiperoxidase (PAP) method (24), for light microscopy. Whenever feasible, consecutive serial sections were used for the various immunohistochemical reactions and their controls. Autoradiography following administration of 125I-labeled [Tyr3]SP was used for possible identification of SP cells and fibers by uptake mechanisms.

MATERIALS AND METHODS

SP Antiserum. Synthetic SP (Beckman Instruments Company) coupled to succinylated thyroglobulin was suspended in

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Abbreviations: SP, substance P; PAP, peroxidase antiperoxidase.
saline and emulsified in Freund's complete adjuvant supplemented with killed tubercle bacilli. Antiserum against this antigen conjugate was raised in New Zealand White rabbits following multiple intradermal injections (25). The titer of antiserum was determined as reported (26). Multiple bleedings were obtained from each rabbit; all the experiments described here were performed on antiserum obtained on one occasion. Antiserum was used in a dilution of 1:100.

Controls. (i) Inactivated antiserum preincubated for 48 hr with SP in excess (concentration, 100 nmol of SP per ml of antiserum) was used at a dilution of 1:100. (ii) Normal goat IgG (Miles Lab) was used at dilutions of 1:30 or 1:100. (iii) Neurtensin antiserum (27) was used at dilutions of 1:100. (iv) Inactivated neurtensin antiserum preincubated with excess neurtensin (concentration, 200 nmol or 0.7 mg/ml of antiserum) was used at dilutions of 1:100.

Unilateral Dorsal Rhizotomy. Adult rats (Sprague-Dawley, 300 g body weight) were anesthetized with 35% chloral hydrate intraperitoneally (0.1 ml/100 g body weight). The thoracic (T1–T5) segments of their spinal cords were exposed by dorsal laminectomy and the dorsal roots were cut on one side. The animals were killed 24 or 48 hr later. Normal adult rats served as untreated controls.

Preparation of Nervous Tissue for PAP Immunohistochemical Study. Normal untreated and experimental animals were anesthetized with chloral hydrate and perfused through the heart with 1 liter of cold 4% formaldehyde in 0.12 M phosphate buffer (pH 7.5) for 30 min. The tissues were dissected out and left in cold fixative for 30 min to 4 hr and soaked overnight in 30% sucrose/10% formalin. Frozen sections 50 μm thick were cut and mounted in serial order on gelatin-coated slides from 0.05 M Tris-HCl buffer in 0.9% saline, pH 7.6. These sections were allowed to dry in a 60° oven for 30 min to ensure adhesion to the slide. Immunohistochemical reactions were performed by a modification of the unlabeled SP antibody PAP technique. All solutions were diluted in 0.05 M Tris-HCl in 0.9% saline with 1% normal goat serum (pH 7.6) and this buffer was used in all rinses except where stated otherwise. All reactions were carried out at 20° in a humid environment. The sections were rinsed (three times, 2 min each) in buffer, incubated in antiserum (diluted 1:100) for 1–2 hr, rinsed again (three times, 5 min each), and incubated in goat anti-rabbit-IgG (Miles Lab) at a dilution of 1:10 for 30 min. The sections were rinsed again and then incubated with PAP at a dilution of 1:50 for 30–60 min. Following this, they were washed three times with Tris/saline without goat serum and treated with freshly prepared 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Sigma) with 0.1% hydrogen peroxide in 0.05 M Tris-HCl with constant agitation. The sections were rinsed three times in distilled water, dehydrated in ascending alcohol series, cleared in xylene, and mounted unstained in Permount prior to observation with phase-contrast, Nomarski interference, dark-field, and bright-field optics. Regions of positive immunoreactivity were dark brown against a light background. Control sections were carried in parallel throughout, with the first incubations in either normal goat IgG or in antiserum inactivated by preincubation with the appropriate antigen.

Preparation of Tissue for Immunofluorescence Studies. The animals with unilateral dorsal rhizotomies and the nonoperated controls were fixed by vascular perfusion with cold 4% formaldehyde in 0.12 M phosphate buffer. Thoracic spinal cord segments and their sensory ganglia were carefully removed. Tissue from the nonoperated side was kept separate and served as controls. Serial frozen sections 30 μm thick were placed upon gelatin-coated slides with each slide containing separated sections from experimental and control halves of the spinal cord and from each ganglion. This facilitated comparison between control and experimental sections during incubation with antisera and microscopic examination. Separate groups of sections were incubated with SP antiserum or neurtensin antiserum, inactivated SP antiserum preincubated with SP, inactivated neurtensin antiserum preincubated with neurotensin, or normal goat IgG (1:30 dilution). Comparable tissue prepared from untreated rats served as the nonoperated controls throughout these experiments. Dilutions were made with 0.05 M Tris-HCl in 0.9% saline with 1% normal goat serum (pH 7.3) and this buffer was used throughout. The sections were incubated with the antisera and control sera at 37° for 30 min in a humid environment, rinsed three times for 5 min each in the buffer, and subsequently incubated for 30 min at 37° with sheep anti-rabbit-IgG conjugated with fluorescein isothiocyanate (Miles Lab) diluted 1:40. The slides were rinsed in the buffer and cover slipped in glycerine–Tris/saline (3:1, vol/vol). Cells and fibers stained with fluorescent antibody were examined with a Leitz Orthoplan microscope equipped with a Poem vertical illuminator and a xenon light source. The filters used are described in the order of passage of light: 3-mm BG-38 (red suppression filter), KP-500 (excitation interference filter), TK-510/515 (built-in dichroic mirror and barrier filters), and K-530 (barrier filter). Objective lenses with high numerical aperture were used to obtain efficient illumination and high resolution (X63 planapo, oil numerical aperture 1.4). Kodak 35-mm Tri-X film with exposures of 0.5–0.1 min was used and developed in Acufine (Acufine, Chicago, IL), which gave an effective ASA rating of 1200.

Autoradiographic Studies with 125I-[Tyr8]SP. (i) 125I-[Tyr8]SP (specific activity 2 μCi/25 nmol) in 50 μl of carbonate buffer (pH 9.9) containing approximately 1 pmol of SP was pulse-injected into the right cerebral ventricle of rats (200 g body weight).

(ii) 125I-[Tyr8]SP with similar specific activity in 10 μl was dripped in vivo directly upon the spinal cord and the sensory ganglia (T1–T4) after careful exposure by dorsal laminectomy and dissection through the connective tissue capsule of the ganglia. After 3 hr, both sets of animals were perfused with aldehyde mixtures (1% glutaraldehyde, 1% formaldehyde in 0.12 M phosphate buffer) and the tissues were frozen-sectioned and prepared for autoradiography with Kodak NTB-2 emulsion (28). Exposures of 6–9 weeks were used, followed by development in D-19 and observation under dark-field illumination.

RESULTS

PAP method

SP Immunoreactivity in Untreated Sensory Ganglia and Spinal Cord. A vast plexus of immunoreactive nerve fibers with varicosities and thin intersegments was observed in Lissauer's tract and the substantia gelatinosa, particularly heavy in lamina I and grading off in intensity to lamina III. Similar fibers were also seen in the region around the central canal and in the neuropil around ventral horn cells. A small number of individual fibers with varicosities were observed traversing the dorsal root between the dorsal horn and the sensory ganglion (Fig. 1).

Immunoreactive SP Cells. Within the sensory ganglia, each 30-μm section displayed approximately 12 immunoreactive SP cells and in each cervical ganglion there was a total of approximately 250 SP cells. These cells were generally small, averaging 10-20 μm in diameter, and were fusiform or stellate in shape. Several lay in the area immediately beneath the
FIG. 1. (A and B) Control thoracic ganglia. SP-immunoreactive cells (large arrows) labeled by the PAP method are recognized by their small size (10-20 μm) compared to the larger unlabeled sensory ganglion cells (asterisks) and by accumulations of granular material. A more homogeneous, nongranular, less intense PAP immunoreactivity is seen in lamellae (small arrows) between ganglion cells (asterisks) and surrounding blood vessels (b). (Nomarski interference optics, SP antiserum diluted 1:100; ×900.)

(C) Control and (D) ipsilateral dorsal rhizotomy. SP immunoreactivity is seen in two cells containing numerous intensely fluorescent granules (arrow); they are considerably smaller than the large ganglion cells in the immediate vicinity (g). A less intensely fluorescent, homogeneous reactivity is seen in lamellae surrounding ganglion cells (arrow heads) and near blood vessels in D. (Indirect Coons method, SP antiserum diluted 1:100 and sheep anti-rabbit-fluorescein conjugate; ×900.)
connective tissue capsule of the ganglia; others were scattered throughout the ganglion, singly or in groups of two or three. The most consistent location for many SP cells was among the groups of small cells abutting against vessels of the rich capillary bed of the ganglia.

Careful examination with Nomarski interference contrast optics revealed two varieties of immunoreactivity within the spinal ganglion. (i) Punctate dark-brown granules with sharp contours. These structures were of all sizes from very small and barely resolvable (about 0.1 μm) to quite large (about 3.0 μm) and were generally found packed into cells. An SP-immunoreactive cell invariably contained large accumulations of this material. Occasionally, smaller, individual, punctate dark-brown spots could be observed loose in the area immediately surrounding an SP cell without clear confinement to the cell’s boundaries. Nerve fibers were sometimes observed among ganglion cells; they always bore this dark punctate reaction. (ii) The second variety of immunoreactivity was a smooth, homogeneous, brown reaction, lighter than the dark brown of the punctate SP cellular reaction but darker than the surrounding background. These areas of SP reactivity were not confined to SP cells. Instead, it surrounded the cell or extended as long filament lamellae up to 500–600 μm between satellite cells surrounding ganglion cells. Very often, this homogeneous brown reaction was found around parts of blood capillaries, particularly those near an SP cell. Examples of these two varieties of SP-PAP immunoreactivity are shown in Fig. 1 A and B. 

SP Immunoreactivity after Unilateral Rhizotomy. At 24–48 hr following dorsal rhizotomy the sensory ganglia on the operated side showed an increased number of cells marked by immunoreactivity. Each cell usually contained a larger number of punctate immunoreactive granules of all sizes. There was an increase in the number of immunoreactive nerve fibers; they were thicker than in the nonoperated animal, the reaction product within them was darker, and the varicosities were more dilated. The homogeneous SP reaction also was more obvious. Control material from the nonoperated side and from the untreated controls was comparable and appeared as described above.

Tissues from the experimental, control, and untreated animals treated with normal goat IgG or with SP antisera inactivated by prior incubation with SP in excess showed no reactivity in cells or fibers in the sensory ganglia or in the spinal cord. Similarly, tissues treated with neurotensin antisera showed no neurotensin-reactive cells in the spinal ganglia.

Immunofluorescence method
These observations were made on serial sections of the tissues taken from the unilateral rhizotomy experiments, controls, and untreated animals that were used for the PAP method in order to attempt a close correlation between the results of the two methods. In the tissues from the control side and untreated animals, immunofluorescence was observed in the spinal cord, substantia gelatmosa of the dorsal horn, periaqueductal region, and ventral gray as described for the PAP method. Within the sensory ganglia, a number of cells had intense SP fluorescence and they were similar in size, number, and disposition to those observed after the PAP method. In many fluorescent cells, granular punctate structures were intensely fluorescent (Fig. 1 C and D). No fluorescence was found in the space occupied by the cell nucleus, and there was no particular accentuation of fluorescence in the peripheral cytoplasm of the SP cell [compare with the results reported in cat (22)]. However, in the ganglionic neuropil, there were other immunofluorescent lamellated processes that ran between nonfluorescent elements. Some of these resembled true varicose nerve fibers, whereas others were more suggestive of antigen–antibody fluorescence present in less restrictive structures and an extracellular distribution (Fig. 1 D).

In the sensory ganglia ipsilateral to the dorsal rhizotomy, many more SP-fluorescent cells were found, with numerous intensely granular deposits within their cytoplasm. The ganglionic neuropil also contained larger numbers of nerve fibers, especially within the dorsal nerve roots proximal to the sensory ganglion. The more diffuse SP fluorescence in structures innervating along blood vessel boundaries and around SP cells also increased after rhizotomy. Examples of SP-fluorescent cells in the control animal compared to SP-fluorescent cells, fibers, and other structures found between ganglion cells are shown in Fig. 1 C and D.

'Tissues from the experimental, control, and untreated animals treated with normal goat IgG or with SP antisera inactivated by prior incubation with SP in excess showed no fluorescence in cells or fibers in the sensory ganglia or in the spinal cord. Similarly, these tissues treated with antisera against neurotensin showed no cells in the spinal ganglia.

Experiments with autoradiography following intraventricular pulse injections of 125I-[Tyr8]SP and local application of the radioactive material to the spinal cord and sensory ganglion surfaces in vivo produced no detectable radioactivity over terminals or fibers in the spinal cord and no fibers, cells, or other structures within the sensory ganglia.

DISCUSSION

The two immunohistochemical methods used here show that they have equivalent sensitivities in the display of SP-immunoreactive structures in spinal cord and sensory ganglia. The fluorescence method can be performed more rapidly but the transience of the product makes it impossible to secure permanent preparations for future reexamination. Both methods have confirmed that sensory ganglia contain SP-immunoreactive cells, the processes of which travel to the spinal cord and end in plexuses of SP terminals. When the dorsal roots are severed, SP accumulates in cells and other structures, possibly extracellular, on the ganglion side of the lesion, rendering them more readily detectable. These results substantiate the evidence from earlier experiments (13, 12, 22) in the cat.

The present study documents at the light microscope level that two forms of immunoreactivity exist in SP-containing structures. First, there are granular, intensely SP-reactive, punctate structures of various diameters (0.1–3.0 μm) generally contained within the SP cells but sometimes found dispersed in the ganglion, not confined to the cell body. Second, a more homogeneous and less intense immunoreactivity exists, which is found intercalated between and around ganglion cells and portions of blood capillaries. This material seems to be extracellular. The significance of these discoveries has been elucidated by electron microscopic immunocytochemistry (29). SP-containing structures cannot be demonstrated by autoradiographic methods following exogenous administration of 125I-[Tyr8]SP; apparently there is no uptake system for this radioactive analog of SP. Preliminary studies by the Swedish investigators (Ljungdahl et al., cited in ref. 22) also failed to demonstrate uptake of this SP analog by nerve endings in isolated spinal cord slices. Finally, neurotensin-immunoreactive cells were not observed in spinal sensory ganglia.

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