Ultrastructural identification of substance P cells and their processes in rat sensory ganglia and their terminals in the spinal cord by immunocytochemistry

(granules/storage/release/extracellular/synaptic)

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ABSTRACT The unlabeled substance P (SP) antibody-peroxidase-antiperoxidase reaction was used on tissue prior to embedding in epoxy resins for ultrastructural identification of the SP cell and its immunoreactive granules. The SP cell is 10–20 μm in diameter and has sparse cytoplasm with numerous intensely reactive SP granules 100–300 nm across, large clear vacuoles, elaborate smooth endoplasmic reticulum, fragmentary rough endoplasmic reticulum, dispersed ribosomes, few mitochondria, and a modest Golgi apparatus. The large SP-reactive granules are discharged into the extracellular space, either with cell membrane intact or as unbound dense material. The membrane-bound dense granules are transported intact through endothelial cells into the blood or are picked up by Schwann cells and fibroblasts. Other SP-reactive granules lose their limiting membranes, fragment, and then disperse into fine immunoreactive grains that bind to the extracellular matrix and to collagen. Dispersed SP-reactive granules are transported within myriad pinocytotic vesicles across endothelial cells with numerous luminal plications and are discharged into the blood. Pinocytosis of dispersed SP-reactive material, that can be detected intracellularly, also occurs in Schwann cells and fibroblasts. The SP axons to the substantia gelatinosa are unmyelinated or finely myelinated. Their synaptic varicosities display a generalized axoplasmic immunoreactivity, which also occurs in and around small vesicles. The larger SP synaptic vesicles are intensely reactive.

A previous study (1) using the unlabeled substance P (SP) antibody-peroxidase-antiperoxidase (PAP) method (2) and immunofluorescence demonstrated two varieties of immunoreactivity in spinal sensory ganglia: SP cells with numerous intensely reactive granules 0.1–3.0 μm in diameter, and a less-intense nongranular homogeneous reactivity in lamellae, possibly extracellular, between some large ganglion cells and around blood vessels in the vicinity of SP cells. These cells give rise to varicose fibers that leave the ganglion to travel in the dorsal roots; some of these terminate in the dense SP plexus in the substantia gelatinosa of the spinal cord (see refs. 3 and 4). The present investigation attempted to discover the ultrastructural basis for the two forms of immunoreactivity of SP-positive elements in the sensory ganglia.

Although there is a growing interest in the disposition and functional significance of SP and other neuropeptides, there are few ultrastructural studies on SP axons and no investigation on the SP cell has been reported. Among the few reported studies there appears to be no consensus; it has been reported that SP axons in the substantia gelatinosa of rat spinal cord show PAP immunoreactivity *selectively* in large (60–80 nm) granular vesicles (5), mainly in small vesicles (6), or a combination of these findings with heavy reactivity in large vesicles together

with cytoplasmic reactivity (7). The present study identifies SP-reactive nerve terminals in the spinal cord, describes the SP cell in the sensory ganglia, and provides ultrastructural evidence concerning the transport or release of SP-immunoreactive product at nerve terminals, into the extracellular space, and into the vascular system.

MATERIALS AND METHODS

Preparation of Nerve Tissue for Electron Microscope Immunocytochemistry. Adult normal rats (100 g body weight) anesthetized with chloral hydrate were perfused through the heart with 1 liter of fixative (2% formaldehyde/0.25% glutaraldehyde/0.12 M phosphate buffer, pH 7.3/0.02 mM calcium chloride) for 30 min. Tissue from the cervical spinal cord and cervical and thoracic dorsal root ganglia was dissected out, left in cold fixative for 30 min to 4 hr, and then sectioned at about 10 μm into cold 0.05 M Tris buffer in 0.9% saline (pH 7.6) on a Vibratome (Oxford Instr.). Sections were collected and handled gently thereafter with clean camel hair brushes and placed in nylon mesh wells to facilitate rinsing. Immunocytochemical reactions with a modified PAP method (1) were used on these free sections, which were then embedded in epoxy resin (Epon 812). Antiserum to SP [characterized as described (5)] was used at concentrations of 1:100. Controls were prepared by inactivating the antiserum to SP by preincubation with the antigen in excess and then used at a concentration of 1:100. Normal goat IgG served as a second control at a concentration of 1:30.

After the peroxidase reaction, the free sections were washed in distilled water and then in phosphate buffer with 8% dextrose (for details of electron microscope method see ref. 8, pp. 326–329) and treated with 2% osmium tetroxide for 1 hr, followed by dehydration in ascending methanol series and propylene oxide. The sections were left in propylene oxide and epoxy resin (1:1) for 1 hr, then in the resin alone at room temperature for 1 hr prior to embedding between two sheets of Dacron; for details see ref. 9. After hardening, the sections were examined between the Dacron sheets and selected regions were photographed between glass in the light microscope. Then, thin sections containing selected immunoreactive cells and fibers were cut for electron microscopy. The sections were not stained with any of the heavy metal salts commonly used for electron microscopy. Enhanced contrast in reactive and nonreactive tissues for ease of examination and photography was achieved by operating the electron microscope at lower voltages (e.g., 20–40 kV) and using small objective apertures. The selected region in the 10- μm slice of tissue was serially thin-sectioned from end to end and the sections were placed on slit copper grids. Because of the low penetrance of the reagents, the reac-

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Abbreviations: SP, substance P; PAP, peroxidase-antiperoxidase.

tion of antigen with antibody and the resultant reaction products were limited to approximately 1–3 μm on each surface of the slice. Thus, immunoreactivity was shown by structures through several serial sections on the front and rear surfaces; those in the center of the block were not exposed to the reagents.

RESULTS

The SP Cell. After the PAP reaction, a cell with the following ultrastructural features consistently contained unusual numbers of immunoreactive granules. It was small (10–20 μm in diameter), usually was fusiform or stellate, was covered by a basal lamina, and lacked a satellite cell. The nucleus contained both dispersed and clumped chromatin. The perikaryal cytoplasm, which is generally immunoreactive, showed a dark matrix containing a small number of mitochondria, usually with longitudinally oriented cristae. A conspicuous system of tubular, agranular endoplasmic reticulum, a small Golgi apparatus, fragments of granular endoplasmic reticulum, dispersed ribosomes, and large numbers of pale vacuoles and large vesicles (100 to at least 300 nm in diameter) were typical.

Profiles of two such cells are shown in Figs. 1 *inset* and 2*a*. The cell in Fig. 2*a* has numerous empty vacuoles and appears to have discharged the majority of its granular product. However, the two profiles of cytoplasm from another SP cell, shown in Fig. 1 *inset* and *lower*, display the massive accumulations of immunoreactive material that can be found within large cytoplasmic SP storage vacuoles. Closer scrutiny of one of these vacuoles in the SP cell (Fig. 1 *lower*) shows that it contains discrete large and small masses of granular material, all intensely immunoreactive. The largest vesicular packets are surrounded by small ones, and points of coalescence between them are frequent. The storage vacuole itself has a general immunoreactivity. There is a complex network of smooth endoplasmic reticulum, and granular dense vesicles can be found within the cisternae. The entire cytoplasm of the cell has a fine grainy deposit, each granule being approximately 5–7 nm in diameter. These grains are found in larger accumulations within the storage vacuoles near large immunoreactive dense clumps or finely dispersed over all cytoplasmic organelles except mitochondria (see Fig. 1 *lower*). The mitochondrial membranes, like the other membranes of the SP cell, are immunoreactive but the contents of mitochondria are not.

Discharge of SP Material: Membrane-Bound and Extracellular. Fine varicose processes emanate from the SP cell body (Figs. 1 *inset* and 2) into the surrounding extracellular space between large ganglion cells with their satellite cells and around blood vessels. The surrounding extracellular space has large (100–300 nm) immunoreactive granules (Figs. 2*c* and 3*a–d*), smaller (50–90 nm) fragments of dark immunoreactive material (Fig. 3*a–e*), or a homogeneous, generally dispersed immunoreactivity that appears to be bound to the matrix and collagen fibers (Figs. 1 *inset*, 3*a–e*, and 4).

These electron micrographs suggest that the SP granular immunoreactive material has several fates. These are described in the sequence of electron micrographs that follows.

The granules (100–300 nm in diameter and membrane-bound) occur in the fine processes of the SP cell, distorting the shape of the fiber so that it is almost varicose (Fig. 2*b* and *c*), and the fragments of rough endoplasmic reticulum, mitochondria, and smooth reticulum are pushed aside by these granules (Fig. 2*b*). By a process of cellular fragmentation, observed in serial sections, the entire vesicle is released into the extracellular space (Fig. 2*c*) with membrane intact. Because many of these fibers run immediately adjacent to blood vessels,

the SP granules have rapid access to the blood through the basal lamina and the capillary endothelial cell. This occurs in two forms: either the intact SP-immunoreactive granule is picked up by the endothelial cell (Fig. 2*d*) for ultimate extrusion into the blood (Fig. 2*e*), losing its membrane in the process; or the SP granule fragments and small (40–50 nm across) immunoreactive particles cross the endothelium by pinocytosis.

The process of release and fragmentation of SP granules is shown in Fig. 3. Either SP can be released from the immunoreactive cell as a membrane-bound granule (Fig. 2*c*) or the membrane-free SP reactive product is expelled free into the extracellular space. This leaves the SP cell with a resultant “empty” vacuole (Fig. 3*a*); such vacuoles are as distinctive of SP cells as their massive SP-immunoreactive storage granules. SP granules in the extracellular space, whether membrane-bound or not, proceed to undergo fragmentation. The granule becomes denuded of membrane; the membrane pieces form small, empty, globular entities in the extracellular space (Fig. 3). The SP-reactive granule or core itself breaks down into smaller fragments and the pieces from a single granule can often be traced to their progenitor during this process (Fig. 3*c* and *d*). Eventually, the entire granule and fragments undergo further dissolution (Fig. 3*e*), and immunoreactive product becomes diffusely dispersed in the extracellular space surrounding the SP cell and its major proximal processes (see Fig. 1). The PAP reaction in this case is dispersed, but markedly intense, bound to elements of the extracellular space. This remarkable observation is demonstrated in two micrographs; Fig. 1 *inset* shows the extracellular SP reactivity surrounding a blood capillary and spreading between neighboring Schwann cell-enwrapped ganglion cells. The same area is shown in a serial section with electron microscopic immunocytochemistry (Fig. 1). The two profiles of the SP cell containing large storage granules of SP are imbedded in an extracellular matrix that is intensely and diffusely SP immunoreactive. The neighboring ganglion cells and their satellite cells are free of the PAP reaction. The extracellular matrix laden with SP bulges into the capillary, thinning its wall. The dispersed SP-reactive material can also gain access to the blood through a shuttle of pinocytotic vesicles across the thin endothelium (Fig. 4). These vesicles are laden with finely dispersed SP material on the basal laminar aspect and empty on the luminal aspect of the endothelium. Within each SP pinocytotic vesicle, there are fine (5–7 nm) immunoreactive grains, and the inner aspect of the vesicular membrane is labeled as well. Other cells in the vicinity of the SP cell and the extracellular SP reactive material contain small amounts of immunoreactive material intracellularly. Schwann cells surrounding ganglion cells and axons and some fibroblasts pick up both forms of SP in vacuoles and myriad pinocytotic vesicles.

Controls. Mast cells that have been observed in the sensory ganglia in PAP-treated material are easily distinguished from SP cells by their larger cell size and their uniform, completely non-SP-immunoreactive granules. Control material treated with normal goat IgG and inactivated SP antiserum did not show PAP immunoreactivity.

SP Axons in the Spinal Cord. Unmyelinated and finely myelinated axons with SP reactivity were found in the substantia gelatinosa. Their intervaricose segments were approximately 0.5 μm in diameter, and their varicose portions ranged from 2 to 3 μm in diameter. SP-immunoreactive boutons were found in large numbers. These labeled terminals synapse with dendrites through Gray's type 1 junctions and lie adjacent to nonreactive axonal varicosities. All SP-reactive axons showed a generalized cytoplasmic reactivity in the axoplasmic matrix

FIG. 1. Light (*Inset*) and serial electron micrographs (*Upper* and *Lower*) showing PAP reaction in extracellular space (EX, black arrows) around SP cell cytoplasm (SP, white arrow) with reactive storage vesicles (stv) and empty vacuoles (v). Unreactive ganglion cells (g), Schwann cells (sc), endothelial cells (en), blood capillary (cap), and plications (between arrows) are apparent. A storage vesicle (rectangle in *Upper*) is shown at higher magnification in *Lower*. Large SP-reactive granules (SPg), small granules (white arrow), grains (double arrows), granule within cisterna (black arrow), smooth endoplasmic reticulum (ser), and mitochondria are visible (see text for details). (*Inset*, $\times 900$; *Upper*, $\times 18,000$; *Lower*, $\times 90,000$.)

as well as on membranes bounding organelles and on microtubules, but not within mitochondria. In the terminals, the SP reactivity was also generally in the axoplasm and delineated

the membranes of large and small vesicles. The small vesicles all contained an immunoreactive product that was darker than the generalized cytoplasmic product but less intense than that

FIG. 2. Sequence of electron micrographs showing transport of membrane-bound PAP-reactive SP granules (g or SPg) from cell into capillary lumen. SP cell (a) with reactive granules (g) and empty vacuoles (v) has fine processes (b and c) which contain SP-reactive granules. These are expelled with intact membranes (m) into the extracellular space (ex) (c). Subsequent engulfment by capillary endothelial cell (en) (d) is followed by expulsion of the granule into the capillary lumen with loss of surrounding membrane. Denuded SP granule (SPg) and membranes (m) are detectable in the lumen (cap) (e). (a, $\times 9000$; b, $\times 9000$; c, $\times 19,800$; d, $\times 19,800$; e, $\times 8100$.)

over the granular vesicles. SP terminals varied in their synaptic vesicle content. The smaller vesicles were almost entirely round, and elliptical profiles were rare. The vesicles ranged in diameter from 45 to 60 nm which is larger than the small vesicle popu-

FIG. 3. Sequence of electron micrographs showing vacuolation and fragmentation of PAP-reactive SP granules (SPg). Expulsion of SP-reactive fragments (SPf) can occur, leaving an empty vacuole (v) and loose vesicular membrane in the extracellular space (ex) (a). Intact immunoreactive granules (SPg), fragments (SPf), general reactivity bound to extracellular matrix (SPi), and loose membranes (m) are evidence of fragmentation sequence (b-e). Collagen present in the extracellular space is indicated. (a, $\times 36,000$; b, $\times 19,800$; c, $\times 19,800$; d, $\times 19,800$; e, $\times 27,000$.)

lations of other axons (average, 45 nm) in the neuropil (Fig. 5). The large granular vesicles varied; some axons had only round ones ranging from 90 to 100 nm in diameter and evenly distributed in the axoplasm (Fig. 5). Other axons had larger ves-

FIG. 4. Pinocytosis of SP-immunoreactive material (SPi) from extracellular space (ex) into the endothelial cell (en) through numerous vesicles (white arrows); occasional ones are still attached to the surface membrane. These vesicles, laden with and lined by SPi, pinch off the basal laminar surface; others, devoid of it, are found on the luminal side of the cell. ($\times 45,000$.)

icles, 110–150 nm in diameter, all round, intensely reactive, and clumped together in the peripheral portions of the axon terminal. Certain SP axons had pleomorphic, large, granular vesicles that were elongated or round and ranged in size from 90 nm for the round ones to approximately 200 nm for the long axis of the elliptical ones. Neighboring axons bearing similar vesicles were unlabeled. Controls with normal goat serum and inactivated SP antiserum showed no axons with immunoreactivity.

DISCUSSION

The present study provides ultrastructural immunocytochemical identification of a sensory ganglion cell with SP immunoreactivity, a description of its SP-storage organelles, and the structural correlates for release of SP-reactive granules into the extracellular space in the ganglion. The granules are released intact for transport through the extracellular space and endothelial cell and then into the blood or are released, fragmented, and ultimately broken down into smaller, more elementary particles. These latter are also transported across endothelial cells for ultimate discharge into the blood. SP granules and fragments have been observed within other cellular elements such as Schwann cells and fibroblasts in the ganglion, taken up from the extracellular space as intact granules or in dispersed pinocytotic vesicles. This evidence suggests that the SP cell may be a source of extracellular SP for neighboring cells and for dispersal through the blood.

SP cells project to the spinal cord via the dorsal roots and terminate in a vast plexus of axons with variable morphologic features in their large synaptic vesicles. SP is carried in greatest concentration in large vesicles but the axoplasm and smaller vesicles also have SP reactivity. All SP-reactive axons observed so far have both forms of labeled vesicles and reactive axoplasm. There may be heterogeneous populations of SP-reactive axons as evidenced by the presence of different sizes of large granular vesicles. SP-immunoreactivity has not been observed either in large vesicles exclusively or in small vesicles in axons that are not generally reactive. This evidence is not in complete accord with the conclusions of other investigators (5–7). Nevertheless, the ultrastructural findings of all these studies are supported by biochemical investigations, using subcellular fractionation techniques, on central nervous tissue (10–13) and peripheral

FIG. 5. Two profiles of SP-immunoreactive axons (SP Ax) in the substantia gelatinosa of the spinal cord of a rat. A light axoplasmic PAP reaction is present generally outlining the small (45–60 nm) vesicles and filling most of them. An intense PAP reaction occurs within the membrane-bound large (90–120 nm) vesicle population. ($\times 36,000$.)

nervous tissue (14). For example, SP values estimated by radioimmunoassay in subcellular fractions prepared from isotonic sucrose homogenates of rat forebrain showed that 66% of the total SP content is recoverable in a crude mitochondrial/synaptosomal fraction and some peptide was recoverable in particulate form in synaptic vesicle fractions (6).

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