Immunohistochemical features of substance P-immunoreactive chromaffin cells and nerve fibers in the rat adrenal gland

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Summary. The distribution of substance P (SP) immunoreactivity and the colocalization of SP with other bioactive substances in chromaffin cells and nerve fibers were investigated in the rat adrenal gland at the light microscopic level. In the capsule and cortex, SP immunoreactivity was seen in some nerve fibers around blood vessels and in thick nerve bundles passing through the cortex directly into the medulla. In the medulla, the SP immunoreactivity was observed in a small number of chromaffin cells; these SP-immunoreactive chromaffin cells were either phenylethanolamine N-methyltransferase (PNMT) immunoreactive or immunonegative, indicating that they were either adrenaline cells or noradrenaline (NA) cells. SP-immunoreactive varicose nerve fibers were also found in the medulla and were in contact with a cluster of the NA cells showing catecholamine fluorescence, which suggests that SP from medullary nerve fibers may regulate the secretory activity of the NA cells. Because no SP-immunoreactive ganglion cell was present in the rat adrenal gland, the intra-adrenal

nerve fibers were considered to be extrinsic in origin. The double-immunostaining method further revealed that the SP-immunoreactive chromaffin cells also exhibit immunoreactivities for calcitonin gene-related peptide (CGRP), and neuropeptide tyrosine (NPY), suggesting that these peptides can also be released from the chromaffin cells by certain stimuli. The intra-adrenal nerve fibers in the medulla were composed of SP-single immunoreactive, and SP/CGRP-, SP/choline acetyltransferase (ChAT)-, SP/nitric oxide synthase (NOS)-, SP/pituitary adenylate cyclase activating polypeptide (PACAP)-, ChAT/NOS-, and ChAT/PACAP-immunoreactive nerve fibers, which may affect the secretory activity of the NA cells. In the adrenal capsule, the nerve fibers were present around blood vessels and showed immunoreactivities for SP/CGRP, SP/NPY, SP/NOS, and SP/vasoactive intestinal polypeptide, suggesting that the origin of nerve fibers in the capsule may differ from those in the medulla.

Introduction

It is well known that the adrenal medulla is composed of two types of chromaffin cells, i.e., adrenaline (A) cells and noradrenaline (NA) cells, and secretes large amounts of catecholamines under the control of preganglionic cholinergic nerve fibers (Ungar and Philips, 1983). For the innervation of the chromaffin cells, cholinergic and non-cholinergic nerves are present in the adrenal gland

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(Parker *et al.*, 1993).

Previous immunohistochemical studies further revealed that chromaffin cells and nerve fibers in the adrenal glands in various mammals contained various enzymes, peptides, and amino acids: tyrosine hydroxylase (TH; Oomori *et al.*, 1994), dopamine beta-hydroxylase (DBH; Oomori *et al.*, 1994), calcitonin gene-related peptide (CGRP; Kuramoto *et al.*, 1987), leu-enkephalin (Leu-ENK; Peltto-Huikko *et al.*, 1985), met-enkephalin (Met-ENK; Schultzberg *et al.*, 1978; Pelt-Huikko *et al.*, 1985), neuropeptide tyrosine (NPY; Kuramoto *et al.*, 1986; Lundberg *et al.*, 1986), pituitary adenylate cyclase activating polypeptide (PACAP; Shiotani *et al.*, 1995; Holgert *et al.*, 1996), substance P (SP; Kuramoto *et al.*, 1985), vasoactive intestinal polypeptide (VIP; Kondo *et al.*, 1986), and gamma-aminobutyric acid (GABA; Oomori *et al.*, 1993). Choline acetyltransferase (ChAT; Holgert *et al.*, 1996) and nitric oxide synthase (NOS; Heym *et al.*, 1994) are also contained in the nerve fibers, but not in the chromaffin cells.

The SP-immunoreactive nerve fibers in the adrenal gland have been recognized as a sensory innervation by immunohistochemistry and tracer experiments (Zhou *et al.*, 1991b; Heym *et al.*, 1995). However, it is still unclear which chromaffin cells (A or NA cells) contain SP, whether or not SP-immunoreactive nerve fibers show special distribution in the adrenal medulla, or which type of bioactive substances colocalize with the SP-immunoreactive nerve fibers in the adrenal medulla. The present study examined SP-immunoreactive cellular and neural elements in the rat adrenal gland by immunohistochemistry in order to investigate these queries. Light microscopic immunohistochemistry combined with histofluorescence microscopy was used to determine the chromaffin cells with SP immunoreactivity and those innervated by the SP-immunoreactive nerve fibers. The double-immunostaining technique was used to determine the colocalization of SP and other substances such as CGRP, ChAT, Leu-ENK, Met-ENK, NOS, NPY, PACAP, phenylethanolamine N-methyltransferase (PNMT), and VIP.

Materials and Methods

Twenty five male Wistar rats (8 weeks old; body weight 180–200 g; Japan SLC, Shizuoka) were used in this study. They were anesthetized with diethyl ether and then perfused with 100 ml of physiological saline and 80 ml of 4% paraformaldehyde or 0.5% glutaraldehyde/4% paraformaldehyde in 0.1 M phosphate buffered saline

(PBS; pH 7.3) through the left cardiac chamber. The adrenal glands were removed and immersed in the same fixative for 2–4 h. After rinsing in PBS, the adrenals were immersed overnight in 30% buffered sucrose. Subsequently, they were frozen and cut into 12 μ m thick sections on a cryostat. These sections were mounted on glass slides coated with poly-L-lysine (P-1399, Sigma, St. Louis, MO, USA).

Immunohistochemical analysis was performed by the avidin-biotin-peroxidase (ABC) method using a staining kit (PK-6100) purchased from Vector Laboratories (Burlingame, Stillwater, CA, USA). The sections were incubated overnight with a rabbit SP antiserum (dilution 1:5000, No. 20064, DiaSorin, MN, USA) at 4°C followed by incubation in biotinylated goat anti-rabbit IgG and ABC complex for 1 h at room temperature. Antigen-antibody reaction sites were visualized by incubating with diaminobenzidine tetrahydrochloride (Dojin Chemical, Kumamoto) and 0.01% hydrogen peroxide in a 25 mM Tris-HCl buffer (pH 7.3).

To detect a formaldehyde-induced fluorescent reaction, the cryostat sections were examined and photographed using Axioplan 2 fluorescence microscope equipped with a UV filter (No. 2). Fixation with 0.5% glutaraldehyde added to 4% paraformaldehyde is suitable to demonstrate noradrenaline (NA) fluorescence in the adrenal medulla (Falck and Torp, 1961). After being photographed for NA fluorescence, the same sections were immunostained with the rabbit SP antiserum.

For double-immunostaining, the sections were incubated with a mixture of two primary antisera (Table 1) raised in different species for 12 h at 4°C. The immunoreacted sections were rinsed in PBS and then incubated with cyanine (Cy2)-conjugated or indocarbocyanine (Cy3)-conjugated antisera (Table 1) for 2 h at room temperature. After PBS washing, the slides were coverslipped in glycerol/PBS and observed under an Axioplan2 fluorescence microscope using filter No. 10 for Cy2-labelled specimens and filter No. 20 for Cy3-labelled specimens.

To clarify the relationship between SP and other substances in the nerve fibers in the rat adrenal gland, we examined the colocalization ratio of SP/other substances—SP/CGRP, SP/ChAT, SP/NOS, and SP/PACAP immunoreactivities—and of each substances—ChAT/NOS, ChAT/PACAP, ChAT/CGRP, NOS/CGRP, and PACAP/CGRP immunoreactivities—in the nerve fibers. At the microscopic level, we randomly selected at least 10 fields of view (40x object lens) of the adrenal medulla (excluding the cortical area) from at least 10 sections obtained from each of five animals. The selected fields were photographed using a digital camera (COOLPIX

5000; Nikon, Tokyo). Adobe Photoshop Elements (version 2.0; Adobe Systems, San Jose, CA, USA) and Scion Image Beta 4.02 Win (Scion Corporation, Frederick, MD, USA) were used to view the photographed images and measure the unit area (0.03 mm²). The number of each substance-immunoreactive nerve fiber was counted and calculated for the colocalization ratio in the unit area.

Results

In the adrenal cortex, no SP immunoreactivity was seen in the cortical cells (10–15 µm in diameter), while some nerve fibers showed immunoreactivity for SP. These SP-immunoreactive nerve fibers were surrounded with blood vessels and ran between the capsule and zona glomerulosa. Thin SP-immunoreactive nerve fibers also

Table 1. List of primary antisera and secondary fluorescence conjugated antisera used for immunohistochemistry in the present study.

Primary antisera	Host animals	Dilution	Catalogue No.	Source
CGRP	Guinea Pig	1:1000	GHC6009	Peninsula Laboratories Inc, San Carlos, CA, USA
ChAT	Goat	1:125	AB144P	Chemicon International Inc, Temecula, CA, USA
Leu-ENK	Mouse	1:500	4140-0355	Biogenesis Ltd, England, UK
Met-ENK	Mouse	1:500	MAB350	Chemicon International Inc
NOS	Rabbit	1:8000	B220-1	EURO-DIAGNOSTICA, Beijerinckweg, Netherland
NPY	Rabbit	1:5000	6730-0204	Biogenesis Ltd
PNMT	Sheep	1:5000	AB146	Chemicon International Inc
SP	Rabbit	1:1000	20064	DiaSorin, Stillwater, MN, USA
SP	Guinea Pig	1:500	GHC7451	Peninsula Laboratories Inc
PACAP	Rabbit	1:4000	T4469	Peninsula Laboratories Inc
VIP	Guinea Pig	1:5000	GHC7161	Peninsula Laboratories Inc
Secondary antisera	Conjugates	Dilution	Catalogue No.	Source
Anti-goat IgG	Cy3	1:250	705-165-147	Jackson ImmunoResearch, West Grove, PA, USA
Anti-guinea pig IgG	Cy2	1:100	706-225-148	Jackson ImmunoResearch
Anti-guinea pig IgG	Cy3	1:250	706-165-148	Jackson ImmunoResearch
Anti-mouse IgG	Cy3	1:250	715-165-151	Jackson ImmunoResearch
Anti-rabbit IgG	Cy2	1:100	711-225-152	Jackson ImmunoResearch
Anti-rabbit IgG	Cy3	1:250	711-165-152	Jackson ImmunoResearch
Anti-sheep IgG	Cy3	1:250	713-165-147	Jackson ImmunoResearch

CGRP: calcitonin gene-related peptide; ChAT: choline acetyltransferase; Leu-ENK: leu-enkephalin; Met-ENK: met-enkephalin; NOS: nitric oxide synthase; NPY: neuropeptide tyrosine; PNMT: phenylethanolamine N-methyltransferase; SP: substance P; PACAP: pituitary adenylate cyclase activating polypeptide; VIP: vasoactive intestinal polypeptide; Cy2: cyanine; Cy3: indocarbocyanine

Fig. 1. Light micrograph of SP immunoreactivity in the rat adrenal gland. SP-immunoreactive nerve fibers are seen in the cortex and medulla. Scale bar = 200 μ m. **Inset** shows a highly magnified micrograph of a SP-immunoreactive cell in the medulla. Co: cortex, Me: medulla. Scale bar = 20 μ m

coursed in thick nerve bundles passing through the cortex directly into the adrenal medulla (Fig.1).

In the adrenal medulla, there were very few SP-immunoreactive chromaffin cells (10–15 μ m in diameter; 0–2 cells/section); these SP-immunoreactive cells were found as single cells and contained numerous granules in their cytoplasm (Fig.1 inset). No SP immunoreactivity was seen in the large ganglion cells (60–90 μ m in diameter) in the adrenal medulla.

The SP-immunoreactive nerve bundles divided into thinner bundles to finally become single fibers in the medulla. These SP-immunoreactive varicose nerve fibers were concentrated in some special areas of the medulla and were closely apposed to clusters of chromaffin cells (Fig.1); these immunoreactive nerve fibers were in close contact with the clusters of NA cells that showed catecholamine fluorescence, but were not in contact with A cells (Fig. 2a, b).

In order to examine the colocalization of various bioactive substances in the SP-immunoreactive chromaffin cells and the nerve fibers of the adrenal gland, antisera of various substances were used for double-immunostaining studies. In the adrenal medulla, SP-immunoreactive chromaffin cells were either immunoreactive or immunonegative for PNMT. The SP-immunoreactive cells also exhibited CGRP or NPY immunoreactivity (Fig.3a–h). There were also CGRP- and NPY-immunoreactive chromaffin cells with PNMT immunoreactivity (data not shown). These cells, polygonal in shape and 10–15 μ m in diameter, had no cytoplasmic process. On the other hand, the SP-immunoreactive chromaffin cells showed no ChAT, Leu-ENK, Met-ENK, NOS, PACAP, or VIP immunoreactivities.

Double-immunostaining also revealed that some SP-immunoreactive nerve fibers showed CGRP, ChAT, NOS, or PACAP immunoreactivities in the rat adrenal medulla

Fig. 2. Histofluorescence micrograph of the rat adrenal medulla showing noradrenaline (NA) fluorescence (**a**) and a light micrograph of SP immunoreactivity of same section (**b**). SP-immunoreactive nerve fibers are in close contact with clusters of NA cells, but not adrenaline cells. Scale bar = 60 μ m

(Fig.4–7). These fibers ran among the chromaffin cells and formed a basket-like network around these cells. Table 2 shows the ratio of the colocalization of SP, CGRP, ChAT, NOS, and PACAP immunoreactivities in the nerve fibers of the rat adrenal medulla. The percentages of SP/CGRP-double-immunoreactive and SP-single-immunoreactive

nerve fibers in the total SP-immunoreactive nerve fibers were 38% and 62%, respectively, and a few CGRP-single-immunoreactive fibers were also observed. The percentages of SP/ChAT-double-immunoreactive and SP-single-immunoreactive nerve fibers in the total SP-immunoreactive nerve fibers were 56% and 44%,

Table 2. The ratio of the colocalization of SP, CGRP, ChAT, NOS, and PACAP immunoreactivities in the nerve fibers of the rat adrenal medulla.

Antisera (A/B)	Numbers of A- and B-immunoreactive nerve fibers (C)	Total numbers of A-immunoreactive nerve fibers (D)	Ratio of C/D (%)
SP/CGRP	1,270	3,353	38
SP/ChAT	1,689	3,013	56
SP/NOS	1,597	2,790	58
SP/PACAP	2,339	2,725	86
ChAT/NOS	1,410	1,468	96
ChAT/PACAP	830	1,051	79

Fig. 3. Double immunofluorescent micrographs showing SP (**a**, **c**, **e**, **g**), PNMT (**b**, **d**) CGRP (**f**), and NPY (**h**) immunoreactivities in the rat adrenal medulla. A SP-immunoreactive cell (**a**, **c**; arrow) is also immunoreactive for PNMT (**b**; arrow) or immunonegative for PNMT (**d**; arrow head). A SP-immunoreactive cell (**e**, **g**; arrow) also exhibits CGRP (**f**; arrow) or NPY (**g**; arrow) immunoreactivity. Scale bars = 30 μ m

Fig. 4 and 5. Double immunofluorescent micrographs showing SP (**4a**, **5a**), CGRP (**4b**), and ChAT (**5b**) immunoreactivities in the rat adrenal medulla. Some SP-immunoreactive nerve fibers (**4a**, **5a**) also show CGRP (**4b**) and ChAT (**5b**) immunoreactivities (arrows) while others are SP-only-immunoreactive (arrowheads). Scale bars = 40 μ m

respectively; the percentages of SP/NOS-double-immunoreactive and SP-single-immunoreactive nerve fibers in the total SP-immunoreactive nerve fibers were 57% and 43%, respectively; and the percentages of SP/PACAP-double-immunoreactive and SP-single-immunoreactive nerve fibers in the total SP immunoreactive nerve fibers were 86% and 14%,

respectively. There was also a large number of ChAT-, NOS-, or PACAP-single-immunoreactive nerve fibers. However, none of the SP-immunoreactive fibers exhibited Leu-ENK, Met-ENK, NPY, or VIP immunoreactivities in the rat adrenal medulla. We further examined the colocalization ratio of each substance contained in the SP-immunoreactive nerve fibers in the

Fig. 6 and 7. Double immunofluorescent micrographs showing SP (**6a**, **7a**), NOS (**6b**), and PACAP (**7b**) immunoreactivities in the rat adrenal medulla. Some SP-immunoreactive nerve fibers (**6a**; arrows, **7a**) also show NOS (**6b**; arrows) and PACAP (**7b**) immunoreactivities while others are SP-only-immunoreactive (arrowheads). Scale bars = 40 μ m

adrenal medulla (Fig.8–9). The percentages of ChAT/NOS-immunoreactive nerve fibers in the total ChAT-immunoreactive nerve fibers were 96%, and those of ChAT/PACAP-immunoreactive nerve fibers in the total ChAT-immunoreactive nerve fibers were 79%. However, no ChAT/CGRP, CGRP/NOS, or CGRP/PACAP-immunoreactive nerve fibers were observed in the rat

adrenal medulla.

In the adrenal capsule and cortex, the SP-immunoreactive varicose nerve fibers were formed around blood vessels and immunoreactive for CGRP, NPY, NOS, or VIP (Fig.10a–h). No ChAT-, Leu-ENK-, Met-ENK-, or PACAP-immunoreactive nerve fibers were observed around the blood vessels in the capsule or cortex.

Fig. 8 and 9. Double immunofluorescent micrographs showing ChAT (**8a, 9a**), NOS (**8b**), and PACAP (**9b**) immunoreactivities in the rat adrenal medulla. Almost all ChAT-immunoreactive nerve fibers (**8a, 9a**) show NOS (**8b**) and PACAP (**9b**) immunoreactivities. Scale bars = 40 μ m

Discussion

The existence of a very few SP-immunoreactive chromaffin cells in the adrenal medulla has been previously reported in various mammals (Kuramoto *et al.*, 1985; Heym *et*

al., 1994, 1995), but it was uncertain whether the SP-immunoreactive cells were A cells or NA cells. The present study confirmed that a small number of SP-immunoreactive chromaffin cells were present in the adrenal medulla of the rat and demonstrated that they were either PNMT-immunoreactive or -immunonegative,

Fig. 10. Double immunofluorescent micrographs showing SP (a, c, e, g), CGRP (b), NOS (d), NPY (f), and VIP (h) immunoreactivities in the rat adrenal capsule and superficial cortex. Some SP-immunoreactive nerve fibers (a, c, e, g) also exhibit CGRP (b), NOS (d), NPY (f), and VIP (h) immunoreactivities (arrows) around the blood vessels. V: blood vessel, Scale bars = 30 μ m

Fig. 11. Schematic illustration of SP immunoreactivities in the rat adrenal gland. SP-immunoreactive materials are shown in both A cell and NA cell. The origin of the SP/other substances-immunoreactive nerve fibers may have at least two pathways — sensory ganglia and other ganglia. The nerve fibers innervating the blood vessels of capsule and chromaffin cells are from different origins. A: adrenaline, Ach: acetylcholine, CGRP: calcitonin gene-related peptide, NA: noradrenaline, NO: nitric oxide, NPY: neuropeptide tyrosine, SP: substance P, PACAP: pituitary adenylate cyclase activating polypeptide, V: blood vessel, VIP: vasoactive intestinal polypeptide

suggesting that SP may be synthesized in both A cells and NA cells. Our double-immunostaining data further revealed that the SP-immunoreactive chromaffin cells also showed CGRP or NPY immunoreactivities in the rat adrenal medulla, and all CGRP- and NPY-immunoreactive chromaffin cells also showed PNMT immunoreactivities. Previous immunohistochemical studies have maintained that the expressions of CGRP and NPY immunoreactivities were only found in the A cells (Kuramoto *et al.*, 1986, 1987; Kondo *et al.*, 1986; Lundberg *et al.*, 1986), while Met-ENK, Leu-ENK, and PACAP immunoreactivities appeared in the A and/or NA cells of the adrenal medulla (Schultzberg *et al.*, 1978; Shiotani *et al.*, 1995). These results suggest that SP and other peptides may be synthesized in both types of chromaffin cells and are released from the granules by exocytosis under appropriate stimuli.

The close apposition of SP-immunoreactive nerve fibers to chromaffin cells found in this study may affect the secretory activity of the chromaffin cells. Previous immuno-electron microscopic observations revealed that SP-immunoreactive nerve endings formed synaptic contacts with chromaffin cells (Kuramoto *et al.*, 1985).

Previous physiological studies also reported that SP had inhibitory effects on catecholamine secretion by the stimulation of nicotinic receptor agonists in bovine chromaffin cells and rat adrenal gland although the single use of SP had no effect on chromaffin cells (Livett *et al.*, 1979; Mizobe *et al.*, 1979; Boksa *et al.*, 1982; Nieber and Oehme, 1987; Richter *et al.*, 1989; Zhou *et al.*, 1991a; Krause *et al.*, 1997; Suzuki *et al.*, 1999). Some other reports also showed that SP prevented against the desensitization of the nicotinic response (decrease of catecholamine secretion) of the bovine chromaffin cells (Boksa and Livett, 1984; Khalil *et al.*, 1988). These results suggest that SP from intra-adrenal nerve fibers has an inhibitive or protective effect on the chromaffin cells in the rat adrenal medulla. In the present study, we further found that the SP-immunoreactive nerve fibers were mainly in contact with the NA cells, but not with the A cells in the rat adrenal medulla, indicating that SP from intra-adrenal nerve fibers may exert effects mainly on the secretory activity of NA cells, but not that of A cells. This finding is supported by a previous physiological study showing that SP inhibited the release of NA to a greater extent than that of A (Krause *et al.*, 1997).

Our double-immunostaining studies showed the presence of SP/CGRP- (38%), SP/ChAT- (56%), SP/NOS- (57%), and SP/PACAP-immunoreactive nerve fibers (86%) in the rat adrenal medulla. There were also nerve fibers showing ChAT/NOS immunoreactivities (96%) and ChAT/PACAP immunoreactivities (79%). Judging from the ratio of ChAT and NOS immunoreactivities in the nerve fibers, the colocalization ratio of NOS/PACAP immunoreactivities (about 80%) is similar to that of ChAT/PACAP immunoreactivities (79%). Thus, we consider that these substances may be released together with SP under the appropriate stimuli and may have secretory or inhibitory effects on the chromaffin cells. In fact, some previous studies reported that acetylcholine, NO, CGRP, and PACAP affect on the adrenal chromaffin cells: catecholamine release by acetylcholine (Douglas and Poisner, 1965), the inhibition of catecholamine release by NO (Barnes *et al.*, 2001), the downregulation of the nicotinic receptor function by CGRP (Giniatullin *et al.*, 1999), and catecholamine release by PACAP (Fukushima *et al.*, 2001).

Since no ganglion cells with SP immunoreactivity were found in the rat adrenal gland, the SP-immunoreactive nerve fibers in the adrenal gland are regarded as extrinsic in origin. Previous studies using retrograde tracing methods revealed that the nerve fibers innervating the adrenal medulla arose from the sensory neurons of the dorsal root ganglia and nodose ganglia (Mohamed *et al.*, 1988; Zhou *et al.*, 1991b; Parker *et al.*, 1993; Heym *et al.*, 1994; Dun *et al.*, 1996). It was also reported that the neurons of the dorsal root ganglia and nodose ganglia contained various substances: CGRP (Helke and Hill, 1988; Heym *et al.*, 1995; McCarthy and Lawson, 1997), NOS (Aimi *et al.*, 1991; Heym *et al.*, 1995; Ding *et al.*, 1998), PACAP (Moller *et al.*, 1993; Dun *et al.*, 1996), and SP (Helke and Hill, 1988; Zhou *et al.*, 1991b; Heym *et al.*, 1995; McCarthy and Lawson, 1997). These results suggest that the SP-single-, SP/CGRP-, SP/NOS-, and SP/PACAP-immunoreactive nerve fibers shown in this study are derived from the sensory ganglia described above.

In this study, we demonstrated that some SP-immunoreactive nerve fibers also expressed ChAT immunoreactivity, although there is no report of the neuron showing ChAT immunoreactivity in sensory ganglia. Because ChAT-immunoreactive nerve fibers contained abundant NOS (96%) and PACAP (79%), some SP/NOS- and/or SP/PACAP-immunoreactive nerve fibers may come from ganglia other than the sensory ones. Previous studies showed the presence of SP/ChAT-, ChAT/NOS-, and NOS/PACAP-immunoreactive neurons in the sphenopalatine, the otic, the internal carotid, and/or the ciliary ganglia (Hardebo *et al.*, 1992; Wang *et al.*, 1993; Uddman *et al.*, 1999) as well as the presence of SP/ChAT- and ChAT/NOS-immunoreactive neurons in the pedunclopontine and the laterodorsal tegmental nuclei of the brainstem (Standaert *et al.*, 1986; Sutin and Jacobowitz, 1990; Sugaya and McKinney 1994). Other previous studies showed the presence of SP/ChAT- and ChAT/NOS-immunoreactive neurons in the intestine (Sang and Young, 1998; Li and Furness, 1998, 2000). Taken together, these findings indicate that the SP-immunoreactive nerve fibers in the rat adrenal gland may be derived not only from the sensory neuron, but also from the other neurons mentioned above, although the neural connection between the adrenal medulla and these neurons has yet to be established. Figure 11 shows the possible origins of SP- and other substances-immunoreactive nerve fibers innervating the adrenal gland.

The present study also showed that a few SP-immunoreactive varicose nerve fibers ran along blood vessels in the capsule and cortex. Thus, the SP-immunoreactive nerve fibers apposed to blood vessels may exert vasodilatory effects on these vessels in the rat adrenal capsule, as previous studies revealed that SP had a vasodilatory effect (Pernow, 1981). It should be noted that SP-immunoreactive nerve fibers also showed immunoreactivities for CGRP, NOS, NPY, and VIP in blood vessels in the capsule, while SP-immunoreactive fibers exhibited immunoreactivities for ChAT, CGRP, NOS, and PACAP in the medulla. Similarly, Oomori *et al.*, (1994) reported different patterns of the colocalization of tyrosine hydroxylase, dopamine beta hydroxylase, NPY, and VIP in the nerve fibers in the capsule and the medulla of the rat adrenal gland. These results imply that the origins of the immunoreactive nerve fibers in the capsule are different from those in the medulla. Physiological studies have demonstrated the vasodilatory effects of NO, SP, CGRP, and VIP (Pernow, 1981; Thulin *et al.*, 1984; Fischer and Born, 1985; Ignarro *et al.*, 1987), and the vasoconstriction effect of NPY (Petty *et al.*, 1984), suggesting that NO, SP, CGRP, NPY, and VIP may be released from the intra-adrenal nerve fibers around blood vessels and exert a vasomotor effect in the rat adrenal capsule and cortex.

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