Distribution of Substance P-Containing and Catecholaminergic Nerve Fibers in the Rabbit Carotid Body: An Immunohistochemical Study in Combination with Catecholamine Fluorescent Histochemistry*

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Summary. The distribution of substance P (SP)-immunoreactive nerve fibers in the rabbit carotid body was studied in combination with catecholamine autofluorescence images of sections where SP immunoreactivity was confirmed. Immunoreactivity for SP was found in nerve fibers distributed in the parenchyma of the carotid body. No glomus cells with SP immunoreactivity were observed in the carotid body. On comparing the distribution of SP-immunoreactive fibers with the catecholamine autofluorescence image in a single section, most SP fibers appeared associated with the fluorescent glomus cells, and were located around clusters of them. These results support the suggestion that SP fibers in the cat and rat carotid bodies are involved in chemosensory mechanisms. Furthermore, a survey of the present results and previous ones reported by other workers indicates that SP may be an essential neuropeptide in chemoreceptor organs in most vertebrates from amphibians on upwards evolutionally. In addition, the courses of some catecholaminergic fibers precisely agreed with those of some SP fibers. This suggests that certain sympathetic nerve fibers also contain SP.

In the vertebrates from amphibians and upwards along the evolutionary scale, a pair of carotid bodies exists at the carotid bifurcation (ADAMS, 1954). They function as peripheral arterial chemoreceptors sensitive to changes in the partial pressure of blood gasses (PO2 and PCO2) and to the hydrogen ion concentration of the arterial blood supply (ISHII et al., 1966; BISCOE, 1971). These chemoreceptors are important for the regulation of the respiratory and cardiovascular systems, and are richly supplied with nerve fibers. In mammals and amphibians, they are innervated by the carotid/sinus nerve, a branch of the ninth cranial nerve, i.e., the glossopharyngeal nerve (ROGERS, 1963; ISHII and ISHII, 1973; VERNÄ, 1979), and in birds by branches originating from the distal vagal ganglion and the recurrent laryngeal nerve (JONES and PERVES, 1970; ABDEL-MAGIED and KING, 1978; KAMEDA et al., 1988).

In the last decade, substance P (SP), which was originally studied in sensory systems, has been immunohistochemically demonstrated in the nerve fibers and the glomus cells in the carotid body of humans (SMITH et al., 1990) and seven experimental species: the cat (LUNDBERG et al., 1979a; CUELLO and MCQUEEN, 1980; WHARTON et al., 1980; YATES and CHEN, 1987; SCHEIBNER et al., 1988; PRABHAKAR et al., 1989), guinea pig (KUMMER et al., 1989a), rat (HELKE et al., 1980; JACOBOWITZ and HELKE, 1980; YATES and CHEN, 1987), chicken (KAMEDA, 1989), frog, salamander, and newt (KUSAKABE et al., 1991, 1993, 1994; KUSAKABE, 1992). There is, however, no immunohistochemical evidence regarding the rabbit carotid body. This may be because most commercial antisera for neuropeptides are raised in the rabbit. Information as to whether SP exists within the carotid body of the rabbit would aid in understanding the significance

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of chemoreceptor mechanisms from a comparative viewpoint.

In the present study, the rabbit carotid body was immunostained with anti-SP serum raised in a rat, and studied with the catecholamine fluorescence images of the same sections that were immunostained with SP antiserum. The results were compared with those previously reported in other vertebrate species.

MATERIALS AND METHODS

Five adult male rabbits weighing 2.0–2.5 kg were used. Under sodium pentobarbital (50 mg/kg) anesthesia, the animals were perfused intracardially with heparinized (1 IU/ml) 0.1 M phosphate buffer saline (PBS), pH 7.4, followed by freshly prepared 4% paraformaldehyde in 0.1 M PBS, pH 7.4. The carotid bifurcations of both sides were surgically exposed and removed from the body. The carotid bodies with surrounding tissues were then carefully cut out under a dissecting microscope, and immersed in the same fixative for an additional 6–8 h at 4°C. After a brief washing with PBS, the specimens were cryoprotected in 30% sucrose in PBS. The specimens were then cut serially at 20 μm on a cryostat, and mounted on poly-L-lysine coated slides. These sections were processed with the indirect immunofluorescence method for demonstrating SP immunoreactivity. After washing in several changes of 0.3% Triton X-100 in 0.1 M PBS (PBST), the sections were treated for 30 min with a protein blocking agent (Immunon) at room temperature to block nonspecific protein binding sites. Then they were cut serially at 20 μm on a cryostat, and mounted on poly-L-lysine coated slides. These sections were processed with the indirect immunofluorescence method for demonstrating SP immunoreactivity. After washing in several changes of 0.3% Triton X-100 in 0.1 M PBS (PBST), the sections were treated for 30 min with a protein blocking agent (Immunon) at room temperature to block nonspecific protein binding sites. Then they were incubated overnight at 4°C in SP monoclonal antibody (1:2,000) raised in a rat (Sera Lab). After rinsing in 3 changes of PBST, the sections were incubated with rhodamine-conjugated goat anti-rat IgG (Cappel, 1:100) for 2 h at room temperature. The specimens were then rinsed and mounted in a glycerine-paraphenylenediamine mixture. First, the immunofluorescence originating from rhodamine was examined with an Axiovert 35 microscope (Zeiss). Then the catecholamine fluorescence excited by formaldehyde was observed with the same microscope. For fluorescent images, an HBO 100 super high pressure mercury lamp (Osram), an exciting filter system (546 nm for rhodamine, 395–440 nm for catecholamine), and an emission filter system (590 nm for rhodamine, 470 nm for catecholamine) were attached to the Axiovert microscope. For reference to the general histology, the sections in which immunofluorescence and catecholamine fluorescence were detected were stained with hematoxylin and eosin after washing out the mounting medium. As a control, sections were incubated overnight in the preabsorbed serum with synthetic SP (50 μM).

RESULTS

In sections stained with hematoxylin and eosin, the carotid body was found to be composed of clusters of glomus cells, capillaries, and small nerve bundles (Fig. 1A). Many SP-immunoreactive nerve fibers appeared throughout the parenchyma of the carotid body (Fig. 1B). The immunoreactive fibers were recognized as fine processes with some varicosities. In the cross sections of small nerve bundles, many fibers were immunoreactive for SP (Fig. 1C). No glomus cells with SP immunoreactivity were recognized.

When the area showing the SP immunoreactivity (Fig. 2A) was examined with the catecholamine fluorescence system, many glomus cells appeared intensely fluorescent (greenish yellow) (Fig. 2B). Single and clustered glomus cells were distributed throughout the carotid body. A moderate number of fine fluorescent catecholaminergic fibers (greenish yellow) were also found between fluorescent glomus cells (Fig. 2B), and were associated with the arterioles and small arteries surrounding the carotid body. It would seem that these catecholaminergic fibers are sympathetic fibers, as suggested by Verna (1975).

When the distribution of SP fibers was compared with that of the fluorescent glomus cells and catecholaminergic fibers in the same section: 1) most SP fibers with varicosities were associated with the clusters of fluorescent glomus cells, and some clusters were encircled by SP fibers; 2) the location of some catecholaminergic fibers precisely agreed with that of some fine SP fibers; and 3) there was no fluorescence originating from catecholamine in the small nerve bundles (Fig. 2A, B). To clarify the relation between SP fibers and glomus cells, and between SP and catecholaminergic fibers, tracings of these three structures from Figures 2A and 2B are shown together in Figure 2C.

DISCUSSION

The present study demonstrated that the immunoreactivity to SP was localized in the nerve fibers distributed in the parenchyma of the rabbit carotid body as previously demonstrated in the carotid body of other animal species. As shown in Table 1, SP-immunoreactive nerve fibers can be widely found in the carotid bodies of all seven species hitherto
Fig. 1 A. Cryostat section of the rabbit carotid body (CB) stained with hematoxylin and eosin after detecting SP immunofluorescence. Two rectangles indicate the areas in B and C. ECA external carotid artery, N nerve bundles. Bar = 100 µm. B. SP-immunoreactive fibers in the parenchyma of the carotid body. Bar = 50 µm. C. SP fibers in the small nerve bundle. Bar = 50 µm.
Fig. 2. A and B show the same area of a frozen section of the rabbit carotid body. The large arrow in A, B and C points to the same cluster of glomus cells. A. Substance P (SP) immunofluorescent image. Many thread-like fibers with baricosities are distributed near a capillary and glomus cells. An arrowhead indicates a cross section of small nerve bundle. B. Catecholamine (CA) fluorescent image. Many glomus cells with intense fluorescence are visible. Fine catecholaminergic fibers are located near these fluorescent cells. C. A composite trace drawn on the basis of A and B at the same magnification. Dotted and solid lines indicate SP-immunoreactive and catecholaminergic fibers, respectively. At the three arrows, SP fibers encircle a cluster of glomus cells (black masses), and at the two double arrowheads, the location of SP fibers coincides with that of catecholaminergic fibers. Bar=100 μm.
examined, except for humans, and the rabbit in this study. In the cat and rat, it is thought that SP fibers are involved in chemoreception (Lundberg et al., 1979a; Helke et al., 1980; Jacobowitz and Helke, 1980; Wharton et al., 1980). Figure 2C, in which most SP fibers are located near the clusters of glomus cells that have been considered to be chemoreceptor cells, with some of them encircling the clusters, strongly supports this suggestion in cat and rat. In the amphibians (frog, newt, and salamander), we have suggested that SP fibers participate in the vascular regulation of the carotid labyrinth through the smooth muscle cells richly distributed in the subendothelial stroma of the labyrinth (Kusakabe et al., 1991, 1993, 1994; Kusakabe, 1992). In birds (chicken), Kameda (1989) has suggested that SP fibers might be involved in both the regulation of chemoosensory activity and the carotid body blood flow. Although it appears that the role of the SP fibers in the chemoreceptor organ differs in each species, it seems possible that SP is an indispensable neuropeptide in the nerve fibers supplying the chemoreceptor organs in most vertebrate species from amphibians upwards.

On the other hand, the present study showed for the first time that the catecholaminergic (sympathetic) fibers also contain SP in the chemoreceptor organs. Figure 2C, in which the agreement of the tracing of some catecholaminergic and SP fibers is clearly shown, helps to clarify this. The coexistence of two different messenger substances, such as a neuropeptide and the classical neurotransmitters, catecholamine and acetylcholine, has been demonstrated in the vascular system (Lundberg et al., 1982; Edvinsson et al., 1983) and other autonomic nervous systems (Lundberg et al., 1979b, 1980; Anglade and Tsuji, 1990). Kummer et al. (1989b) have suggested that autonomic nerve fibers exert their effect on chemoreception mainly via vascular mechanisms. In the case of the coexistence of a neuropeptide and a classical neurotransmitter, it has been generally considered that the neuropeptide modulates the effects of the neurotransmitter (Lundberg et al., 1982; Lundberg and Hökfelt, 1983). A similar relation between these two substances might also be possible in chemoreception.

The nerve fibers associated with the glomus cells in the rabbit carotid bodies originate from the carotid/sinus nerve, a branch of the glossopharyngeal nerve (Verna, 1979), and the sympathetic nerve fibers are derived from the superior cervical ganglion (Verna, 1975). In the present study, some small nerve bundles within and around the carotid body did not show catecholamine fluorescence. This suggests that most

### Table 1. A survey of the immunoreactivity for substance P (SP) in the carotid body in several animal species.

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Filled circles (●) indicate the existence of immunoreactivity for SP, and open circles (○) indicate its absence. Slashes indicate no data.
SP fibers which did not show the coexistence of catecholamine are components of the glossopharyngeal nerve, and that the remaining fibers which did show it might originate from the superior cervical ganglion. On the other hand, KATZ et al. (1983) have demonstrated tyrosine hydroxylase immunoreactivity in the glossopharyngeal petrosal ganglion of the rat. This indicates the possibility that SP fibers which contain catecholamine are also of glossopharyngeal origin. To confirm their precise origin, it will be necessary to perform a denervation experiment.

In conclusion, it seems probable that SP is an essential neuropeptide for arterial chemoreceptor organs in most vertebrate species from the amphibians to mammals. Some catecholaminergic (sympathetic) nerve fibers in the rabbit carotid body also contain SP.

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REFERENCES


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