Invited Review

Peptidergic innervation in the amphibian carotid labyrinth

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Summary. The amphibian carotid labyrinth, which corresponds to the mammalian carotid body and carotid sinus, is innervated by nerve fibers containing substance P (SP), calcitonin gene-related peptide (CGRP), vasoactive intestinal polypeptide (VIP), neuropeptide Y (NPY), FMRFamide (FMRF), and somatostatin (SOM). SP, CGRP, VIP, and NPY immunoreactive varicose fibers are more densely distributed in the peripheral portion of the carotid labyrinth than FMRF and SOM fibers. The time of appearance of SP, CGRP, and VIP is different for each. First CGRP fibers, then SP fibers appear at an early stage of larval development, and finally VIP fibres are detected at a later stage of larval development. Most SP fibres show coexistence with CGRP, and some SP fibres which show coexistence with NPY immunoreactivity are assumed to be continuous with those demonstrating VIP immunoreactivity. This indicates the possibility of coexistence of four different peptides in the same nerve fibers within the labyrinth. In various vasculatures of mammals, it has been shown that SP, CGRP, VIP, and NPY have a vasoactive nature in relation to the vascular smooth muscle cells. On this basis, it seems that the target of the peptidergic innervation in the amphibian carotid labyrinth is the smooth muscle cells which are abundantly distributed in the intervascular stroma. Accordingly, the peptidergic innervation may be involved in the vascular regulatory function of the labyrinth, although the possibility that these peptides participate in the chemoreception cannot be ruled out. In addition, the vascular regulatory function of the labyrinth may be modulated by the interaction of multiple neuropeptides.

Key words: Carotid labyrinth, Neuropeptides, Ontogeny, Coexistence, Immunohistochemistry, Amphibians

1. Introduction

The amphibians have a pair of characteristic vascular expansions at the bifurcation of each common carotid artery into the internal and external carotid arteries (Adams, 1958) (Fig. 1A). The appearance of these expansions is that of a maze-like vasculature (Ishida, 1954; Carman, 1955, 1967a,b; Kobayashi and Murakami, 1975; Toews et al., 1982; Kusakabe, 1990a) (Fig. 1B). For this reason, these expansions have been called the carotid labyrinth. The amphibian carotid labyrinth functions as a peripheral arterial chemo- and baroreceptor sensitive to changes in the partial pressure of the blood gases (PO_2 and PCO_2), in hydrogen ion concentration, and in blood pressure (Ishii et al., 1966). Thus, the carotid labyrinth is considered to correspond to the mammalian carotid body and carotid sinus. This indicates that the carotid labyrinth plays an important role in the regulation of respiratory and cardiovascular systems.

On the other hand, it has long been suggested that the amphibian carotid labyrinth functions in the controlling the blood flow to the internal carotid artery without direct evidence of a mechanism for this (Pischinger, 1934; Boissezon, 1939; Ishida, 1954; Carman, 1955). Ishii and Kusakabe (1982) observed, for the first time, the close apposition of the glomus and smooth muscle cells (g-s connection) in the intervascular stroma of the labyrinth, and the exocytosis of the contents (catecholamines) of dense-cored vesicles at the g-s connection. On this basis, Kusakabe et al. (1987) confirmed physiologically that the carotid labyrinth has a vascular regulatory function through the intervention of the g-s connection (Fig. 5A, B). Thus the multiple functions of the carotid labyrinth underline the importance of this relatively small organ for maintenance of homeostasis and of appropriate blood pressure and blood supply to cephalic regions. Because of these multiple functions, the labyrinth is richly supplied with nerve fibres which originate from the sinus/carotid nerve, a branch of the ninth cranial nerve, i.e., the glossopharyngeal nerve

(Rogers, 1963; Ishii and Ishii, 1973).

Recently, Kusakabe et al. (1991, 1993a, 1994d) observed several immunoreactive neuropeptides in the nerve fibres distributed in the labyrinth, and suggested that the peptidergic innervation may participate in the function of the carotid labyrinth. The ontogeny and coexistence of several neuropeptides in the carotid labyrinth have also been reported by us (Kusakabe, 1992c; Kusakabe et al., 1993c, 1994a). Thus, immunohistochemical studies for neuropeptides in the amphibian carotid labyrinth have so far been performed mainly by our group, although studies on mammals and bird carotid bodies have been done by a number of workers (Kondo and Yamamoto, 1988; Scheibner et al., 1988; Kameda, 1990). In this review, we summarize our recent immunohistochemical studies on the carotid labyrinth after briefly introducing the general morphology of this organ, and finally suggest a possible role of peptidergic innervation in the labyrinth.

2. General morphology and morphogenesis of the carotid labyrinth

General morphology

The structure of the carotid labyrinth in many species of amphibians was first studied using serial sections and reconstruction methods (Ishida, 1954; Carman, 1955, 1967a,b). Thereafter, corrosion casting and scanning electron microscopy were introduced by

Kobayashi and Murakami (1975) to observe the three-dimensional fine structure of the carotid labyrinth in the bullfrog, *Rana catesbeiana*. The carotid labyrinth in anurans is spherical (Fig. 1B) and that in urodele is oblong in shape. This method was very suitable for the analysis of the complicated vascular organization such as the carotid labyrinth, and added several new findings which could not be seen in serial sections and reconstruction methods (Noguchi and Kobayashi, 1977; Toews et al., 1982; Kusakabe, 1990a).

Viewed in toluidine blue-stained sections, the carotid labyrinth in many species of amphibians is composed of a complicated sinusoidal plexus and intervascular stroma to make a complicated maze-like structure (Fig. 2). In the intervascular stroma, the glomus cells (type I cells, chief cells), which are considered to be chemoreceptor cells, are distributed singly or in clusters of 2-4 cells between connective tissues and smooth muscle cells. In fluorescence histochemistry, the glomus cells emit intense fluorescence for biogenic monoamines (Banister and Mann, 1965; Banister et al., 1967; Kobayashi, 1971a; Böck and Gorgas, 1976; Kusakabe, 1990b). In fine structure, the glomus cells are characterized by numerous dense-cored vesicles, 60-120 nm in diameter, in their cytoplasm (Fig. 5A,B). Afferent, efferent and reciprocal synapses are observed on the glomus cells (Rogers, 1963; Ishii and Oosaki, 1969; Kobayashi, 1971b; Poullet-Krieger, 1973; Ishii and Kusakabe, 1982; Kusakabe, 1990b, 1992a,b).
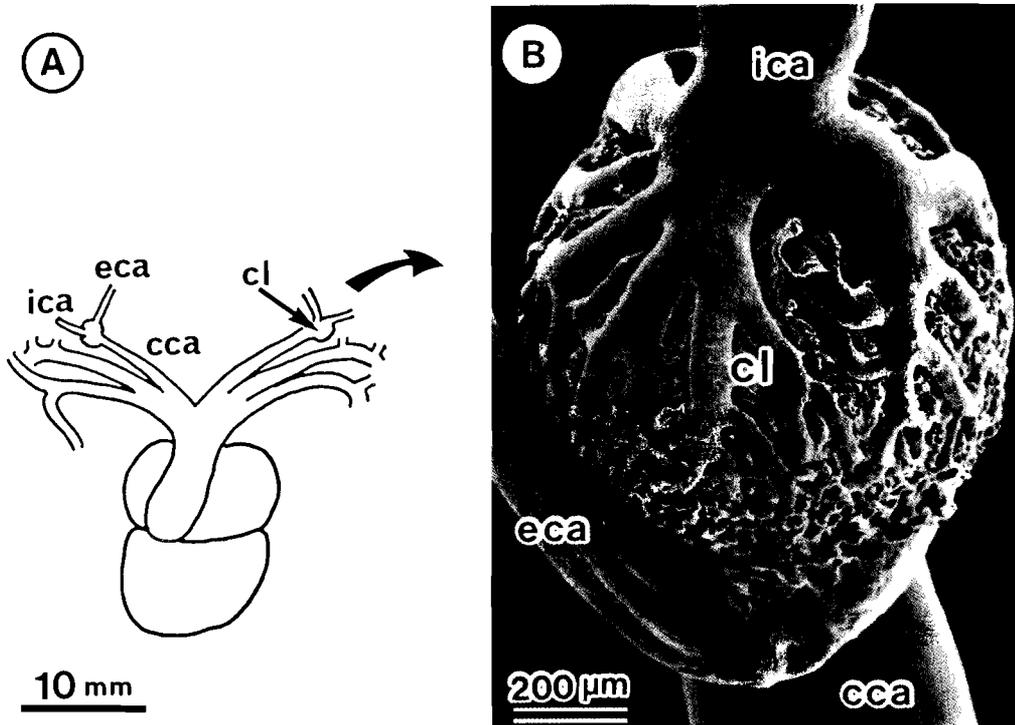
Fig. 1. A. Schematic diagram representing the location of the bullfrog carotid labyrinth. **B.** A scanning electron micrograph of the vascular corrosion casting of the bullfrog carotid labyrinth. cca: common carotid artery; cl: carotid labyrinth; eca: external carotid artery; ica: internal carotid artery. (Kusakabe, 1990a. J. Morphol. vol. 204. Wiley-Liss). x 75

Morphogenesis

In addition to two early observations using serial sections and reconstruction methods (Mishima, 1944a,b), corrosion casting and scanning electron microscopy have also been applied for a precise analysis of the ontogenesis of the carotid labyrinth during larval development and metamorphosis (Kusakabe, 1991b). The morphogenesis of the carotid labyrinth starts at the point where the carotid arch descends to the internal gills (Fig. 3A). The transformation of the appearance of the labyrinth can be summarized in the following six phases. The stages (I-XXV) of larval development and metamorphosis refer to those described by Taylor and Kollros (1946). 1) Through the early stages of larval development (stages I-V), the slightly expanded region of the external carotid artery becomes closely connected with the carotid arch (Fig. 4B-1). 2) By the last of the foot stages (stage XVII), the expanded region becomes globular. 3) At the middle of the metamorphic stages (stage XXII), many protuberances appear on the surface of the globular expansion (Fig. 4B-2). 4) At stage XXIII, these form a rudimentary vascular maze (Fig. 4B-3). 5) At stage XXIV, this globular

expansion is completely surrounded by a simple maze-like structure (Fig. 4B-4). 6) At the final stage of metamorphosis (stage XXV), the carotid labyrinth is nearly completed, and is close to its adult form, as shown in Fig. 1B. From just before the completion of metamorphosis, the forepart of the carotid arch disappears. The carotid arch and the forepart of the external carotid artery are thereafter called the common carotid artery and the internal carotid artery, respectively. To avoid confusion in the terminology, the course of these arteris is shown in Fig. 3B. The morphogenesis of the anuran carotid labyrinth is described schematically in Fig. 4A.

Although corrosion casting and scanning electron microscopy is suitable for the analysis of vascular architecture, no histological information on the structure of the intervascular stroma has been provided. In a recent ultrastructural study on the ontogeny of the carotid labyrinth, the glomus cells appear as early as the initial stages of larval development, and some nerve fibres are close to them (Kusakabe, 1992b). However, these fibres do not show the ultrastructural characteristics of nerve endings. At the middle stages of development, the number of dense-cored vesicles increases remarkably, and some glomus cells show a tendency to form small clusters. At the metamorphic climax, close apposition of the glomus cells and the neighbouring cells, such as smooth muscle cells (g-s connection), endothelial cells (g-e connection), and pericytes (g-p connection), is frequently observed. The g-s connections are more frequently found in juveniles than in larvae (Kusakabe, 1992a) (Fig. 5A,B). Distinct afferent synapses, which are characterized by membrane thickenings with the aggregation of dense-cored vesicles on the glomus cell side, can be found in juveniles (Kusakabe, 1992a), but they cannot be identified in any larval stages (Kusakabe, 1992b). This suggests that the vascular regulatory function through the g-s connection may start at an early stage of the metamorphic climax, and that the chemoreceptor function may begin immediately after metamorphosis.
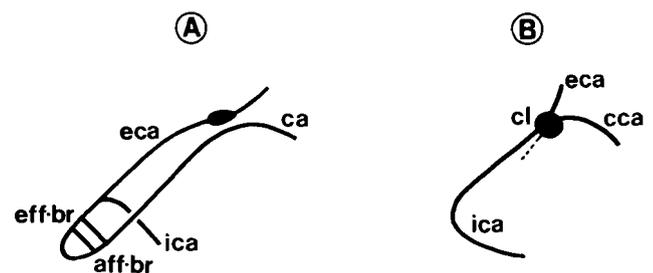

Fig. 2. Semithin cross section of the carotid labyrinth stained with toluidine blue. The carotid labyrinth has a maze-like structure. e: endothelial cell; gc: glomus cell; l: lumen of sinusoid; m: melanophore; sm: smooth muscle cell. x 500

Fig. 3. Schematic illustration showing the organization of the carotid arch before metamorphic stages **(A)** and at metamorphic climax **(B)**. aff.br: afferent branchial artery; ca: carotid arch; cca: common carotid artery; cl: carotid labyrinth; eca: external carotid artery; eff.br: efferent branchial artery; ica: internal carotid artery. (Kusakabe, 1991b. Anat. Embryol. vol. 184. Springer-Verlag).

3. Occurrence and distribution of neuropeptides in the carotid labyrinth

In six amphibian species, immunoreactivity for several neuropeptides has been compared using the peroxidase-antiperoxidase (PAP) method (Kusakabe et al., 1991; Kusakabe et al., 1993a). In four species of anurans *(Bufo Japonicus, Rana castesbeiana, Rana nigromaculata, Xenopus laevis)* and two species of urodelas *(Cynops pyrrhogaster, Ambystoma tigrinum)*, specific immunoreactivity of substance P (SP), calcitonin gene-related peptide (CGRP), vasoactive intestinal polypeptide (VIP), neuropeptide Y (NPY), somatostatin (SOM), and FMRFamide (FMRF) is recognized in the nerve fibres distributed in the intervascular stroma of the carotid labyrinth (Figs. 6-14). There are some differences in the distribution and abundance of immunoreactive fibres, and among the species. The immunoreactive fibres are thin with some varicosities. Generally SP, CGRP, VIP, and NPY immunoreactive fibres are numerous in comparison with SOM and FMRF immunoreactive fibres. These fibres are distributed in the peripheral portion rather than in the central portion of the labyrinth. Most fibres are associated with the sinusoidal plexus, and this distribution pattern has also been confirmed by the differential interference-contrast (Nomarski) images of a section immunostained with the PAP method (Kusakabe et al., 1993a), because the immunoreactive fibres are recognized in relief (Fig. 14A), with most of them located near the sinusoidal wall. The immunoreactive fibres make complicated networks at the divergence of the intervascular stroma, and are often gathered in bundles in the connective tissue surrounding the labyrinth. The distribution of most SP fibres is similar to that of CGRP fibres. SOM fibres are mainly distributed in the connective tissue surrounding the labyrinth. No immunoreactivity for leucine- and methionine-enkephalines (ENKs) is detected in the labyrinth. Frequency of occurrence of these peptidergic fibres in several species of amphibians is summarized in Table 1. Immunoreactive glomus cells for these peptides are not found in the carotid labyrinth.

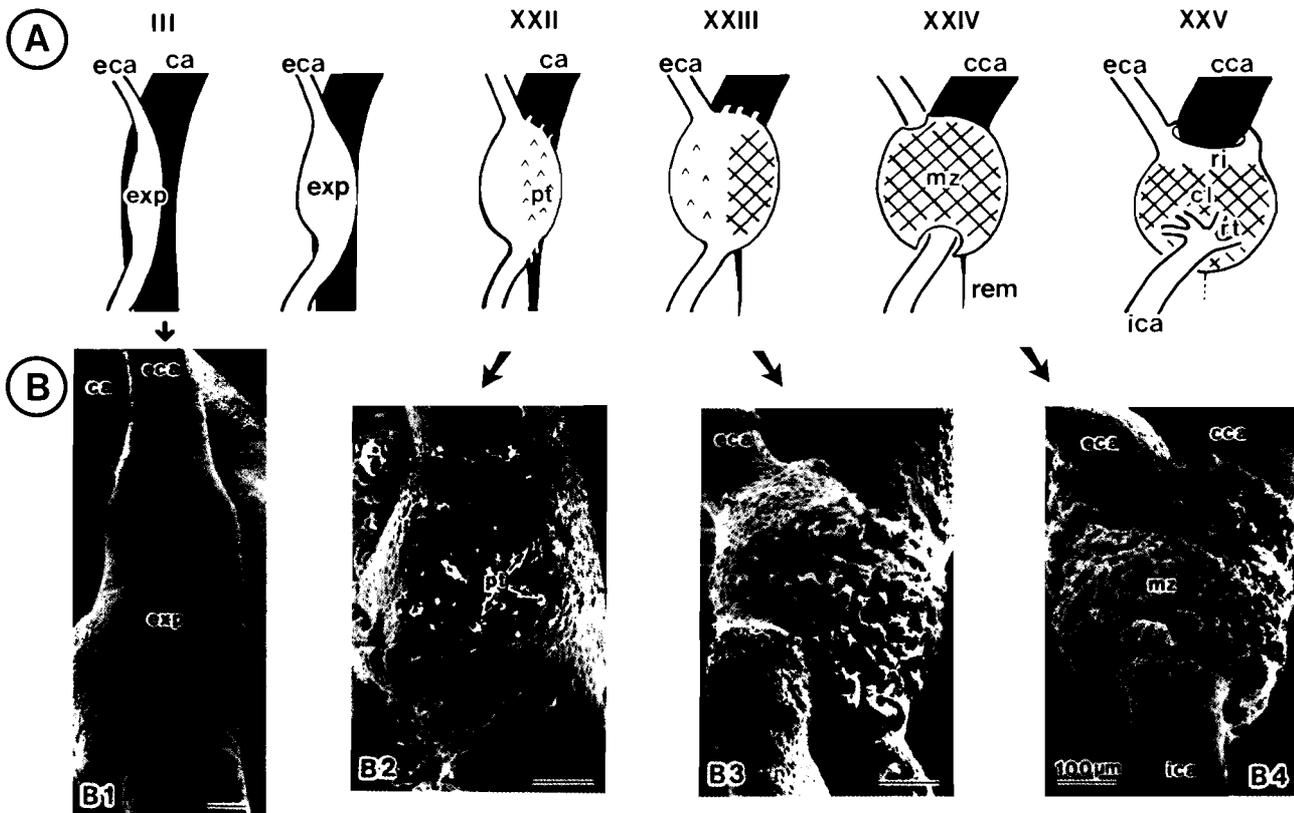In mammals and birds, the immunoreactivity for SP,

Fig. 4. A. Diagram showing the characteristics of the morphogenesis of the bullfrog carotid labyrinth. ca: carotid arch; eca: external carotid artery; exp: vascular expansion; mz: vascular maze; pt: protuberance; rem: remnant vessel; ri: vascular ring; rt: vascular route. **B-1.** Scanning electron micrograph of the vascular corrosion casting of the expansion (exp) of the external carotid artery at the early stage of larval development (stage III). The many elliptical hollows on the surface of the resin cast correspond to the nuclei of endothelial cells. ca: carotid arch; eca: external carotid arch. x 50. **B-2.** Many buds (arrows) on the globular expansion (exp) at stage XXII. x 80. **B-3.** Rudimentary vascular maze (mz) at stage XXIII. ca: carotid arch; eca: external carotid artery. x 80. **B-4.** The globular expansion completely surrounded by the vascular maze at just before the completion of metamorphosis (stage XXIV). x 80. (Kusakabe 1991b. Anat. Embryol. vol. 184. Springer-Verlag).

CGRP, NPY, VIP, and others has been seen in the nerve fibres within the parenchyma of the carotid body (Lundberg et al., 1979a; Wharton et al., 1980; Jacobowitz and Helke, 1980; Kondo et al., 1986; Yates and Chen, 1987; Kondo and Yamamoto, 1988; Kameda, 1989; Kummer et al., 1989; Kusakabe et al., 1994d). Comparing the findings in amphibians with those in mammals and birds, it appears that the nerve fibres innervating the carotid bodies in various vertebrates, from amphibians to mammals, contain many species of
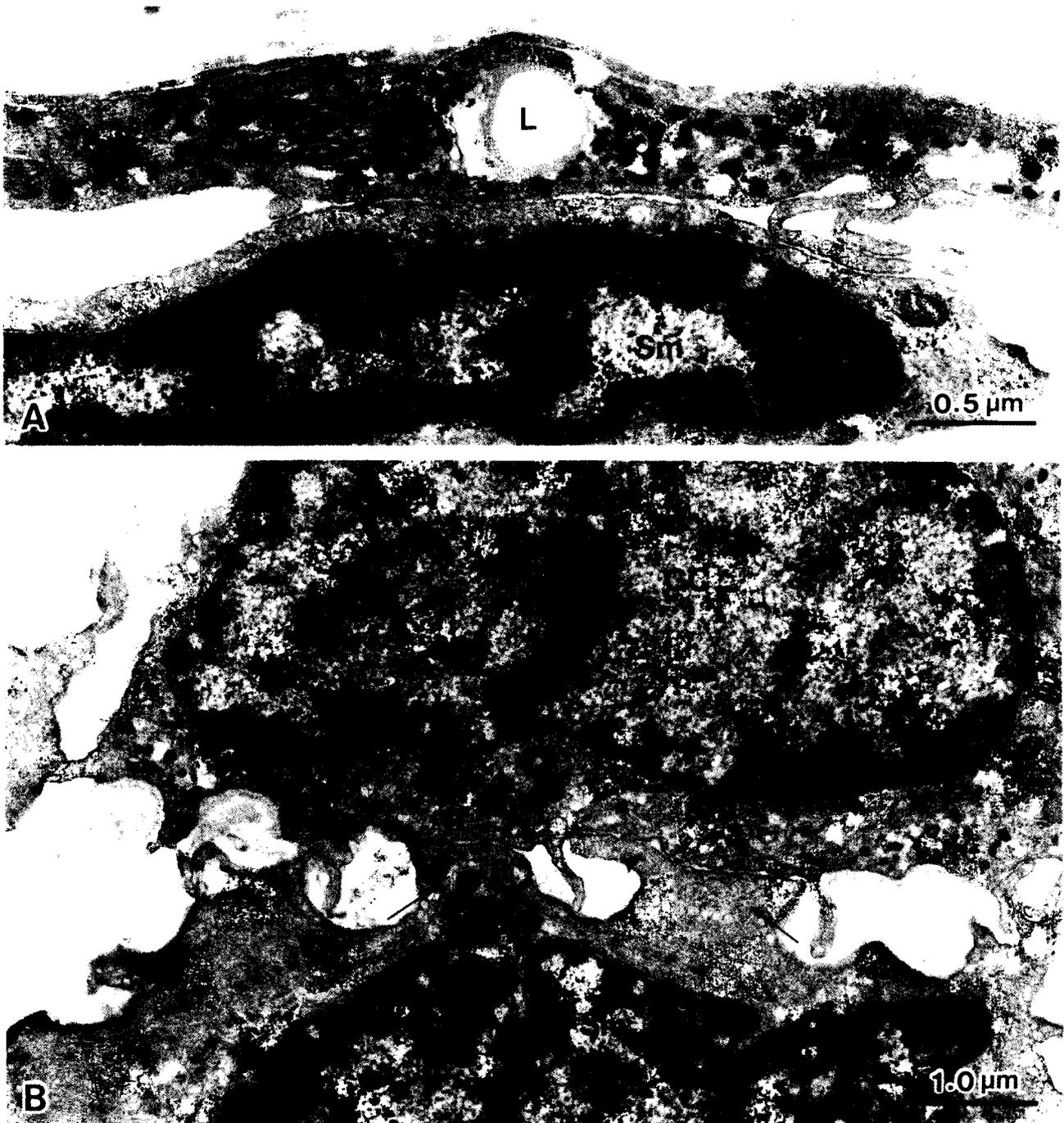

Fig. 5. A. The g-s connection between the thick cytoplasmic process (Cp) of the glomus cell and smooth muscle cell (Sm) in the juvenile bullfrog. L: lipid droplet. x 40,000. B. The g-s connection between the tongue-like projections (arrows) of the smooth muscle cell (Sm) and the glomus cell (GC) in the juvenile bullfrog. An arrowhead indicates the exocytototic figure. C: centriole. x 23,000. (Kusakabe, 1992a. Anat. Embryol. vol. 185. Springer-Verlag).

Table 1. Distribution and relative abundance of immunoreactive nerve fibers of some peptides in amphibian carotid labyrinths.(Kusakabe et al., 1991, Histochemistry, vol. 96, Springer Verlag)

	SP		CGRP		VIP		NPY		SOM		ENKs	
	P	C	P	C	P	C	P	C	P	C	P	C
Bufo japonicus	+++	+++	+++	++	++	++	+	+	+	-	-	-
Rana catesbeiana	++++	++	++++	++	+++	++	++	+	+	-	-	-
Rana nigromaculata	++++	+++	++++	+++	++++	+++	++	++	+	-	-	-
Xenopus laevis	++	+	++	+	+	+	+	+	+	-	-	-
Cynops pyrrhogaster	+++	++	++	+	++	++	++	+	+	-	-	-
Ambistoma tigrinum	+	+	+	+	+	+	+	+	+	-	-	-

Frequency of occurrence of immunoreactive nerve fibers is graded using arbitrary units: -. absent; +, few; ++, moderate; +++, many; ++++, abundant. P: peripheral portion of the carotid labyrinth; C: central portion of the carotid labyrinth.

neuropeptides.

In contrast, there is a difference in the occurrence of peptides within the glomus cells between mammals and amphibians. There is no immunoreactivity for peptides in the amphibian glomus cells. In mammals and birds, however, glomus cells show the immunoreactivity for some peptides: SP in humans and cats (Cuello and McQueen, 1980; Yates and Chen, 1987; Scheibner et al., 1988; Prabhakar et al., 1989; Smith et al., 1990; Wang et al., 1982), VIP in humans (Smith et al., 1990), and ENKs in humans, dogs, and cats (Lundberg et al., 1979a,b; Wharton et al., 1980; Hansen et al., 1982; Varndell et al., 1982; Kobayashi et al., 1983; Smith et al., 1990). This may indicate that the peptide content in the glomus cells varies from species to species.

4. Ontogeny of the neuropeptides in the carotid labyrinth

The ontogeny of SP-, CGRP-, and VIP-containing nerve fibres has been examined in anuran carotid labyrinth by the PAP method (Kusakabe, 1992c). The time of appearance of these three neuropeptides is different for each. At an early stage of larval development (stage II), CGRP-immunoreactive fibres first appear in the wall of the carotid arch and external carotid arteries, and in a thin septa between these two arteries (Fig. 15A). At this stage, SP and VIP fibres are not yet detected (Fig. 15B). SP-immunoreactive fibres first appear in the wall of the arteries and in the septum at stage V (Fig. 16). CGRP and SP fibers appear as a few thin processes with some varicosities. Thereafter, there is no conspicuous change in the distribution and abundance of these two fibers. The tail piece of the larva begins to

regress from around stage XX, and is completely resorbed at stage XXV to finish metamorphosis. At the early metamorphic stage (stage XXII), VIP-immuno-reactive fibers finally appear (Fig. 17). Up to the completion of metamorphosis, the number of these fibers remains low. Through these stages, the morphogenesis of the carotid labyrinth with active angiogenesis is conspicuous, as described above. In spite of this progressive angiogenesis, the relative abundance of CGRP, SP, and VIP fibers does not differ. The schematic diagram in Fig. 4A is helpful to understand the relationship between the ontogeny of the peptidergic fibres and the morphogenesis of the labyrinth. From 1 to 5 weeks after metamorphosis, these fibres increase in number to varying degrees. By 8 weeks after metamorphosis, the distribution and abundance of these three fibres closely resemble those of the adults. Only during the final metamorphic stages do some glomus cells show immunoreactivity for CGRP and VIP. The distribution and relative abundance of SP, CGRP, and VIP immunoreactive fibres and glomus cells in the vascular wall of the two arteries and the carotid labyrinth during metamorphosis and further development is summarized in Table 2.

The ontogeny of the peptide-containing fibers has been studied in the mammalian central nervous system by many workers using immunohistochemistry (e.g., Emson et al., 1979; Pickel et al., 1980, 1982; Shiosaka et al., 1981; McGregor et al., 1982; Palmer et al., 1982; Senba et al., 1982; Yamano et al., 1984). In brief, SP, SOM, and ENK first appear at an early phase of foetal development, and CGRP and VIP appear at a late phase of foetal development and in early postnatal development. In the carotid body, SP-immunoreactive

Figs. 6-9. Cryostat sections of the carotid labyrinth from some species of amphibians, stained by the PAP method with the antisera of several peptides. The many melanophores distributed in the carotid labyrinth are seen as polymorphic dense masses. Bars=100 μm. (Kusakabe et al., 1991, Histochemistry, Vol. 96, Springer Verlag).

Figs. 6 and 7. Substance P (SP)-immunoreactive nerve fibers observed in the carotid labyrinth of *Bufo japonicus* (Fig. 6) and *Rana catesbeiana* (Fig. 7). Dense networks of immunoreactive varicose fibers are distributed in the intervascular stroma of the carotid labyrinth. x 120

Figs. 8 and 9. CGRP-immunoreactive nerve fibers observed in the carotid labyrinth of *Bufo japonicus* (Fig. 8) and *Rana nigromaculata* (Fig. 9). Figs. 6 and 8 are serial sections of the carotid labyrinth. CGRP-immunoreactive fibers show a distribution pattern identical to that of SP-immunoreactive fibers. However, CGRP fibers are less numerous than SP fibers. Fig. 8, x 120; Fog. 9, x 170

6   SP

7   SP

8   CGRP

9   CGRP

100 μm
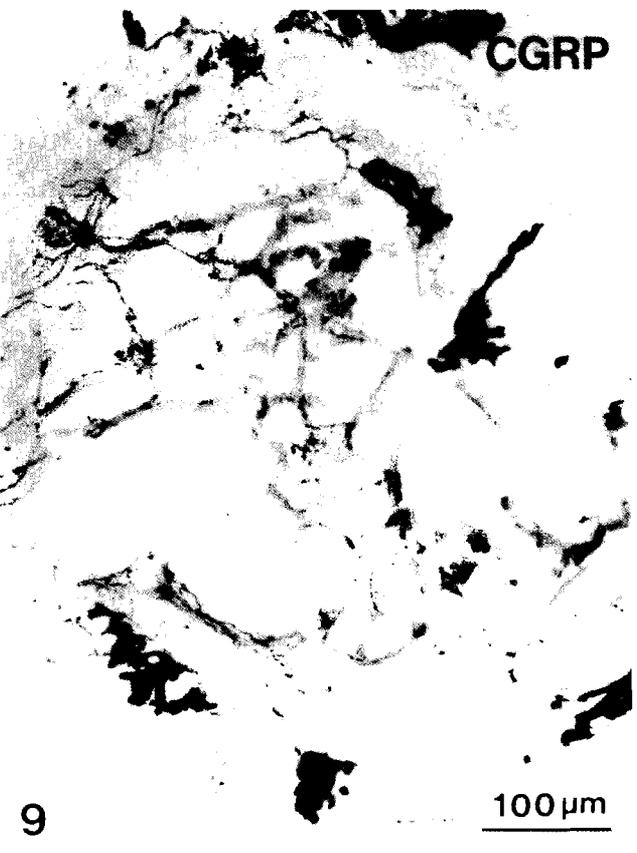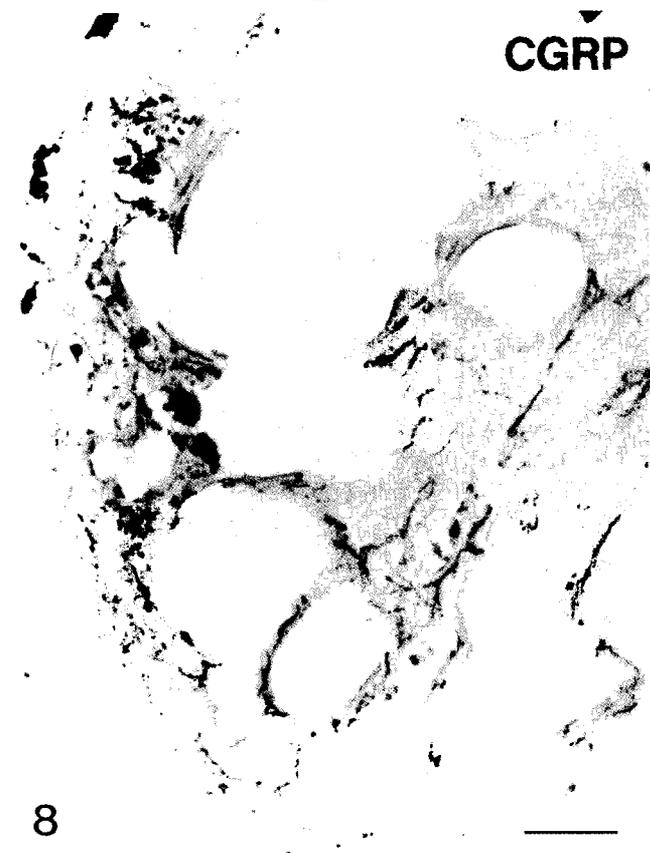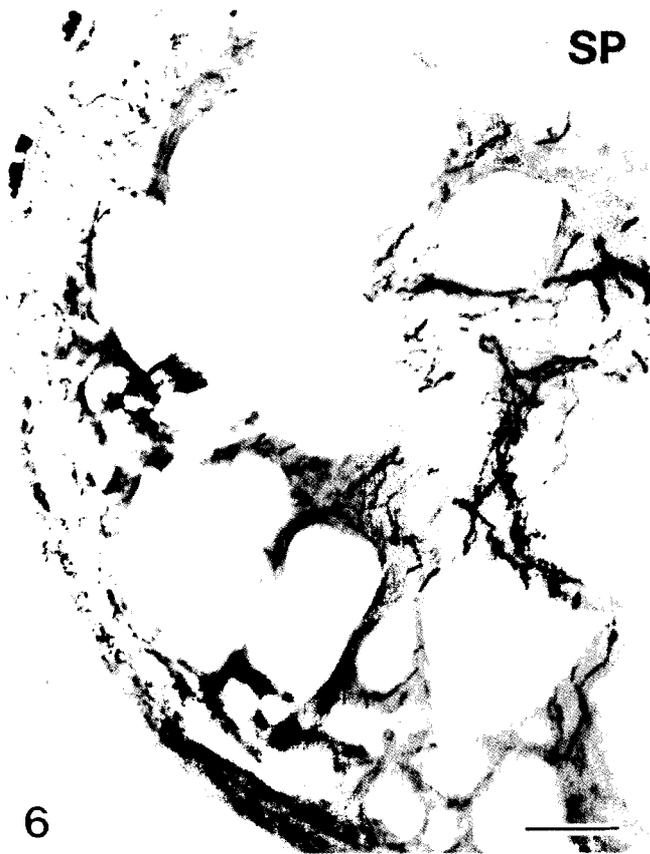
Table 2. Distribution and relative abundance of SP, CGRP, and VIP immunoreactive nerve fibers and glomus cells in the wall of the external carotid artery and the carotid arch, and in the carotid labyrinth during metamorphosis and further development. (Kusakabe, 1992c, Cell Tissue Res., Vol 269, Springer-Verlag).

		STAGES (Taylor and Kollros, 1946)																
		II	III	V	X	XV	XVII	XX	XXI	XXII	XXIII	XXIV	XXV	1W	2W	3W	5W	8W
SP	P	-	-	+	+	+	+	+	+	+	+	+	+	++	++	+++	+++	++++
	C	-	-	+	+	+	+	+	+	+	+	+	+	+	++	++	++	++
	GC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CGRP	P	-	+	+	+	+	+	+	+	+	+	+	+	++	++	+++	+++	++++
	C	-	+	+	+	+	+	+	+	+	+	+	+	+	++	++	++	++
	GC	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-
VIP	P	-	-	-	-	-	-	-	-	+	+	+	+	+	+	++	++	+++
	C	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	++
	GC	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-

Number of immunoreactive nerve fibers and glomus cells is graded using arbitrary units: -, absent; +, few; ++, moderate; +++, many; ++++, abundant. P: vascular wall of the arteries (Stages III-XXIII), and peripheral portion of the carotid labyrinth (Stages XXIV-8W); C: septum between the arteries (Stages III-XXIII), and central portion of the carotid labyrinth (Stages XXIV-8W); GC: glomus cell.

fibres appear before birth in the cat (Scheibner et al., 1988), and CGRP fibers after birth in the rat (Kondo and Yamamoto, 1988), as has been reported in the mammalian central nervous system. Both SP and CGRP fibres in the chicken appear at the early embryonic stage, and VIP fibres at a late embryonic stage (Kameda, 1990). The appearance pattern of SP, CGRP, and VIP in the amphibian carotid labyrinth is similar to that in the chicken carotid body.

The first immunohistochemical detection of SP- and CGRP-containing fibres in the carotid labyrinth is in good agreement with the ultrastructural appearance of nerve fibres (Kusakabe, 1992b). Throughout the larval development and metamorphosis, the relative abundance of SP, CGRP, and VIP fibres remains low, in spite of the organization of the vascular maze and the maturation of the glomus cells (Kusakabe, 1991a,b). Consequently, SP, CGRP, and VIP fibres during larval development and metamorphosis may be nonfunctional, and may start to participate in the function of the labyrinth only after metamorphosis.

Although no glomus cells in the adult carotid labyrinth demonstrate immnunoreactivity for CGRP or VIP (Kusakabe et al., 1991), the immunoreactivity for CGRP and VIP is found transitorily in some glomus cells immediately before and after metamorphosis. This suggests the following two possibilities. One, these peptides may be a factor in growth and differentiation of

the carotid labyrinth as stated by Kameda (1990) for other neuropeptides. Two, CGRP and VIP may provide indirect vascular regulation through the close connection of the glomus and smooth muscle cells at a distance of 10-20 nm (g-s connection), because the g-s connection can be found frequently in the juveniles, and exocytosis of the contents of dense-cored vesicles is frequently seen at the g-s connection (Kusakabe, 1992a).

5. Coexistence of some neuropeptides in the nerve fibres

In the carotid labyrinth, the immunohistochemical coexistence of SP and CGRP was first speculated in two adjacent sections (Kusakabe et al., 1991). Thereafter, this speculation was clarified using double immunohistochemical staining in a single section with anti-SP and anti-CGRP sera against two different animals (Kusakabe et al., 1993c). In the bullfrog carotid labyrinth, almost all SP fibres show coexistence with CGRP (Fig. 18A,B), although a few SP fibres do not show this coexistence. Thus, double immunohistochemical staining for SP and CGRP clearly demonstrates the coexistence of these two peptides in the majority of nerve fibres in the inter-vascular stroma of the carotid labyrinth. The coexistence of these two peptides has also been demonstrated in the guinea pig carotid body (Kummer, 1988). According to this, all SP fibres also exhibit CGRP immunoreactivity,

Figs. 10-13. Cryostat sections of the carotid labyrinth from some species of amphibians, stained by the PAP method with the antisera of several peptides. The many melanophores distributed in the carotid labyrinth are seen as polymorphic dense masses. Bars=100 μm. (Kusakabe et al., 1991. Histochemistry. vol. 96. Springer-Verlag).

Figs. 10, 11. VIP-immunoreactive nerve fibers in the carotid labyrinth of *Rana nigromaculata* (Fig. 10) and *Cynops pyrrhogaster* (Fig. 11). The immunoreactivity is different from species to species. Fog. 10, x 120; Fig. 11, x 200

Fig. 12. NPY immunoreactive nerve fibers in the carotid labyrinth of *Rana nigromaculata*. x 180

Fig. 13. SOM-immunoreactive nerve fibers in the peripheral portion of the carotid labyrinth and in the connective tissues around the labyrinth of *Rana catesbeiana*. x 130

VIP

10

VIP

11

NPY

12

SOM

13

100 µm

and vice versa. These observations in the amphibian carotid labyrinth are not consistent with those in the mammalian carotid body. The mammalian carotid body functions mainly as an arterial chemoreceptor, whereas the amphibian carotid labyrinth functions not only as a chemoreceptor but also as a vascular regulator which controls the blood flow to the cephalic region. The difference in the coexistence of these two peptides between the carotid body and the carotid labyrinth may stem from this. Ultrastructural double-labelling immuno-
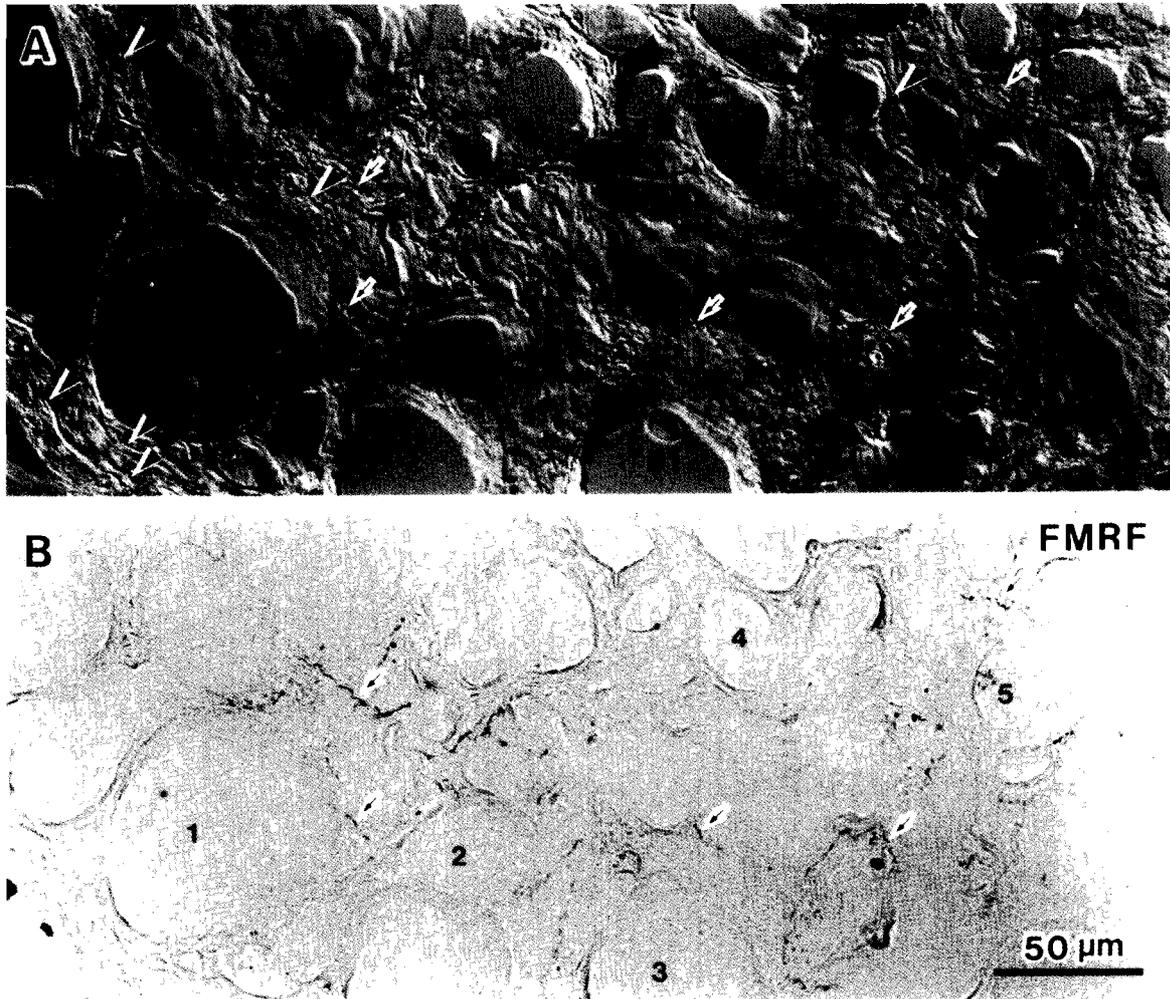

Fig. 14. A. Three-dimensional image showing a cryostat section of the carotid labyrinth taken with a differential interference-contrast (Nomarski) microscope. The labyrinth consists of the sinusoidal plexus (1-5) and intervascular stroma. Across indicate several fine fibres seen in relief. **B.** Cryostat section of A stained by the PAP method with antiserum to FMRFamide. The field corresponds exactly to that of A. A small number of immunoreactive varicose fibres are distributed in the intervascular stroma. The immunoreactive fibres indicated by arrows correspond to the relief fibres in A. Many nerve fibres marked by arrowheads in A were not immunoreactive for FMRFamide in B. x 400. (Kusakabe et al. 1993a. Arch. Histo. Cytol. vol. 56. Daiichi Printing).

Figs. 15-17. Cryostat sections of the expanded region of the external carotid artery and carotid arch, and the carotid labyrinth at various developmental stages, stained by the PAP method with antisera against SP, CGRP, and VIP. The many melanophores distributed in the vascular wall of the arteries and the carotid labyrinth are seen as polymorphic dense masses. (Kusakabe, 1992c. Cell Tissue Res. vol. 269. Springer-Verlag).

Fig. 15. Serial sections of the expanded region of the external carotid artery (eca) and carotid arch (ca) from a stage III larva, stained with CGRP **(A)** or SP antiserum **(B)**. CGRP immunoreactive nerve fibres (arrows) begin to appear at stage III, but SP fibres do not appear yet. x 150

Fig. 16. SP immunoreactive nerve fibres (arrow) first appear in the wall of the external carotid artery (eca) and the carotid arch (ca) at stage V. x 150

Fig. 17. VIP immunoreactive nerve fibres (arrows) appear in the external carotid artery (eca) and the septum with some perforations at stage XXII. ca: carotid arch. x 200

CGRP

eca

ca

15A

SP

eca

ca

15B

SP

eca

ca

16

VIP

eca

ca

17

100 µm
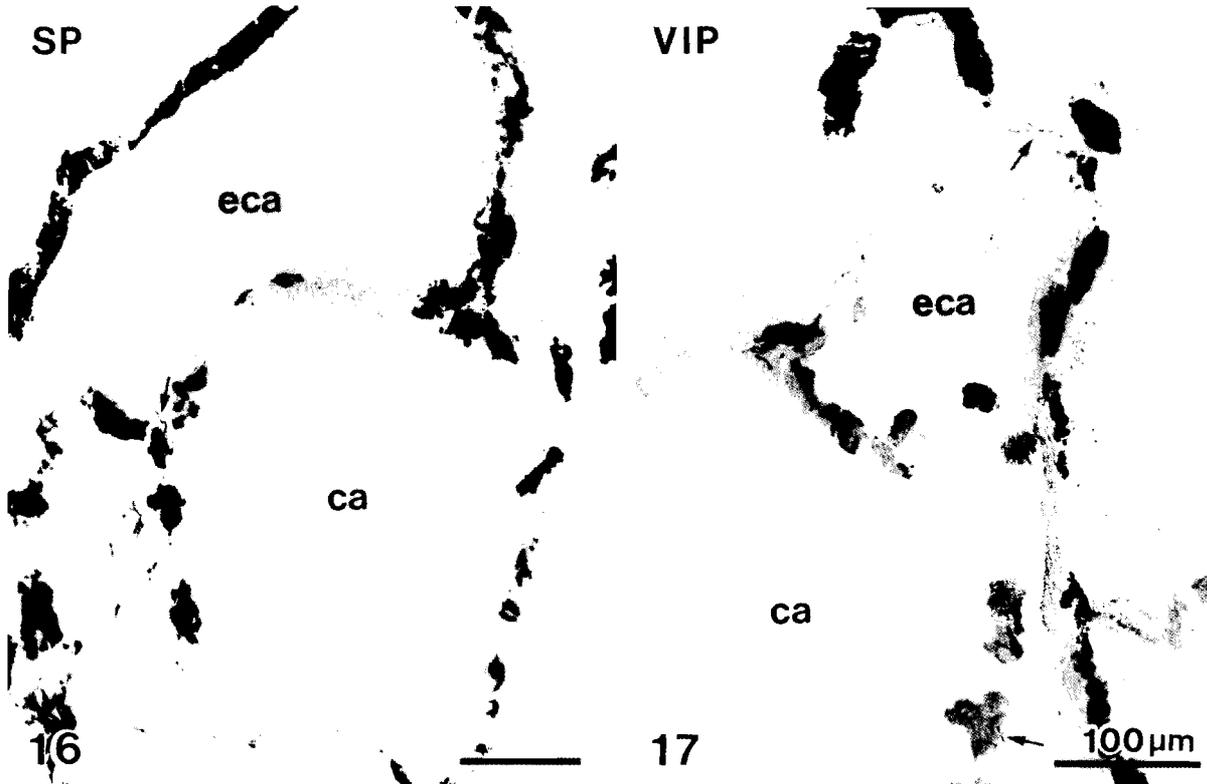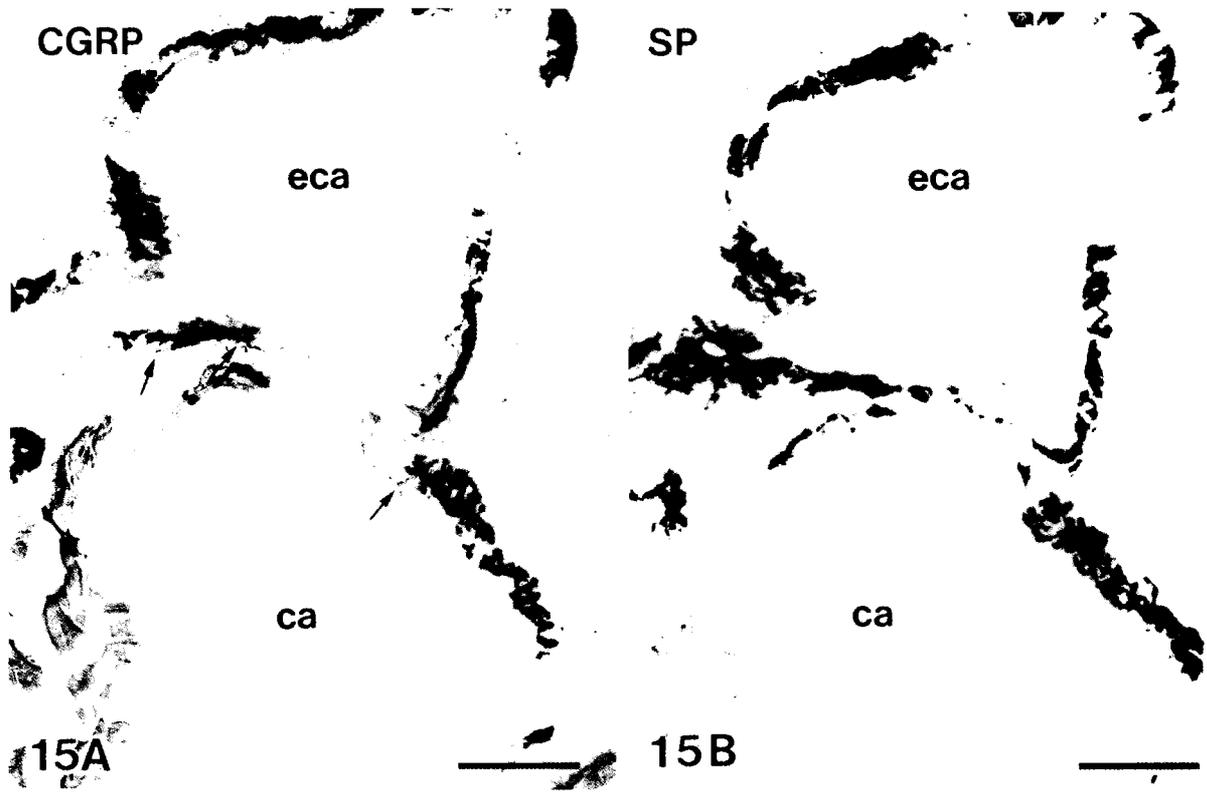
cytochemistry has also demonstrated the subcellular coexistence of SP and CGRP (Kummer et al., 1989). In ultrastructure, SP and CGRP immunoreactivity is found in the same dense-cored vesicles within the axon. In addition, double staining demonstrates the coexistence of SP and FMRF in some fibres within the labyrinth (Kusakabe et al., 1993a).

More recently, the coexistence of SP and NPY, and SP and VIP in the same nerve fibres within the labyrinth has also been suggested in addition to that of SP and CGRP, and of SP and FMRF (Kusakabe et al., 1994a). Approximately one third of SP fibres show the co-

existence of VIP immunoreactivity (Fig. 19A,B), and most SP fibres show the coexistence of NPY immuno-reactivity (Fig. 20A,B). Furthermore the combination of double-labelling immunofluorescence method and alternate consecutive sections confirmed the possible coexistence of SP, NPY, and VIP in a single continuous nerve fibre observed in two serial sections (Kusakabe et al., 1994a). A composite scheme of two adjacent sections is useful to confirm this speculation (Fig. 21). In addition, almost all SP fibres show the coexistence of CGRP immunoreactivity, as previously suggested (Kusakabe et al., 1993c). These findings strongly
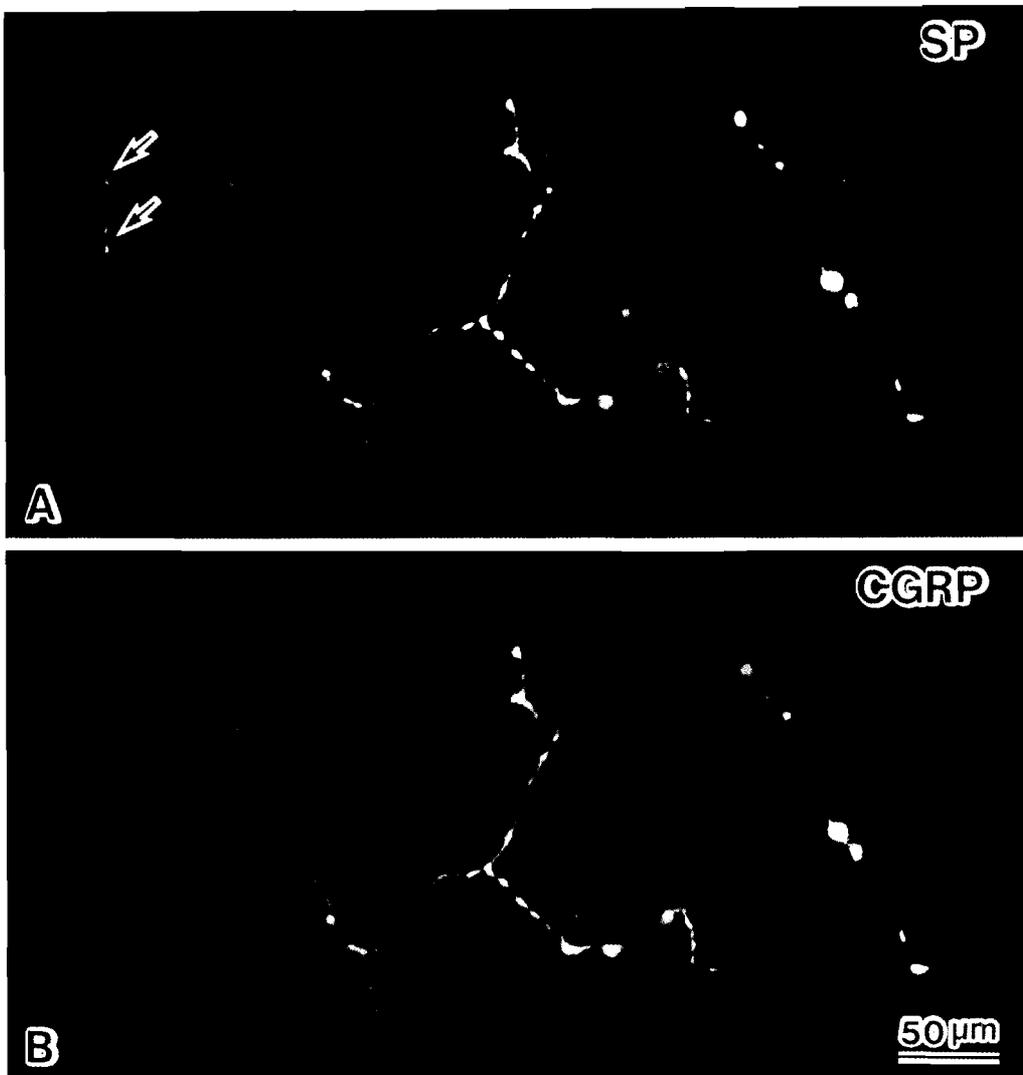

Fig. 18. A and **B** show the same area of a frozen section of the carotid labyrinth. The three asterisks (1-3), which show the lumen of the sinusoid in A correspond to those in B. A and B: Double immunostaining for SP (rhodamine) and CGRP (FITC). The branched varicose fibres are distributed in the intervascular stroma. Most of them are immunoreactive for both SP and CGRP. Arrows show the location of an SP-immunoreactive varicose fibre without CGRP-immuno-reactivity. x 400. (Kusakabe et al., 1993c. Brain Res. vol. 603. Elsevier).

Fig. 19. Double immunostaining for SP **(A)** and VIP **(B)**. Approximately one third of the SP-fibres also demonstrate VIP-immunoreactivity. Arrow, VIP-fibre without SP-immunoreactivity. Three asterisks in A showing sinusoids corresponding to those in B. x 340

Fig. 20. Double immunostaining for SP **(A)** and NPY **(B)**. Approximately one third of the SP-fibres also demonstrate NPY-immunoreactivity. There are two fibres (arrows) with SP but no NPY (A), and two other fibres (arrowheads) with NPY but no SP (B). x 340. (Kusakabe et al., 1994a. Cell Tissue Res. vol. 276. Springer-Verlag).

SP

VIP

19A

B

SP

NPY

20A

B

50 μm
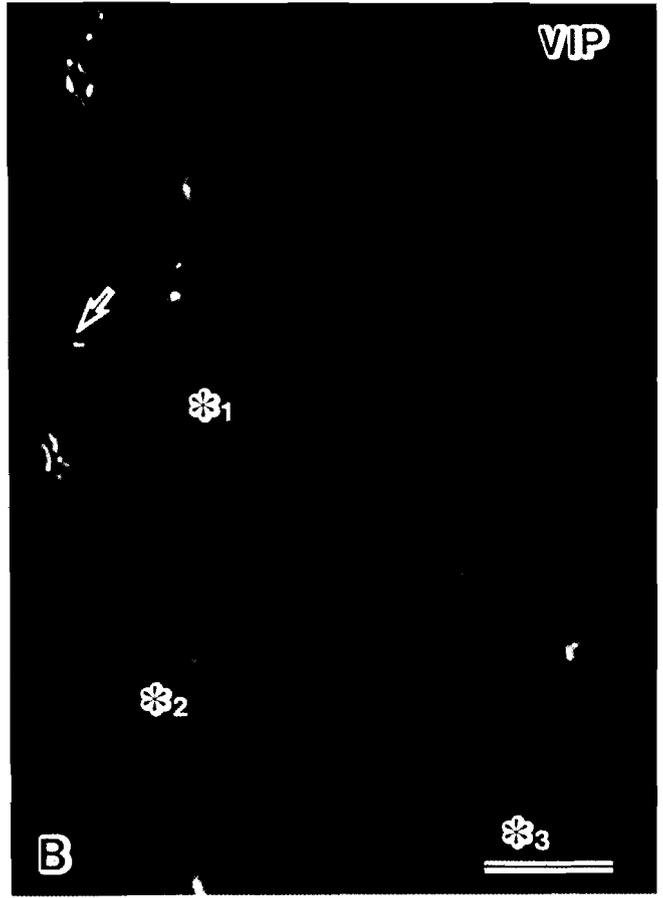
suggest the coexistence of four different neuropeptides, SP, CGRP, NPY, and VIP in the same nerve fibres. The patterns of estimated coexistence of four different peptides are summarized in Table 3.

The coexistence of two different substances, one of the peptides, SP, VIP, and NPY, and one of the classical neurotransmitters, catecholamine and acetylcholine, has been demonstrated in the nerve fibres within various mammalian organs: the submandibular glands (Lundberg et al., 1979b, 1980); the endocrine pancreas (Anglade and Tsuji, 1990a); the myenteric plexus (Anglade and Tsuji, 1990b); and the blood vessels (Lundberg et al., 1982; Edvinsson et al., 1983). Generally, it has been considered that the effect of the neurotransmitter is modulated by the peptide (Lundberg et al., 1982; Lundberg and Hökfelt, 1983). In the coexistence of two different peptides, a similar mechanism has been speculated, but without direct evidence for this (Kusakabe et al., 1993a,c, 1994a).

When we decide about the actual coexistence of two different substances, it is necessary to consider whether an immunoreactive fibre which demonstrates the coexistence is a single axon within a bundle, or whether the two immunoreactivities originate from separate neurons in the same bundle. In fact, some axons are found in bundles in the intervascular stroma of the labyrinth in fine structures (Rogers, 1963; Ishii and Oosaki, 1969; Kobayashi, 1971b; Ishii and Kusakabe, 1982; Kusakabe, 1990b, 1991b, 1992a,b). To clarify this, two devices are proposed. One is an immunohisto-chemical analysis at electron microscopic level as previously reported in the guinea pig carotid body by Kummer et al. (1989). The other is an immuno-fluorescent staining in combination with video-enhanced microscopy as shown by Takenaka et al. (1990). The latter technique is characterized by both high resolution and high contrast detection of fluorescence as an accumulation of differentiated figures. An application of this technique may be useful to the problem of peptide colocalization.

6. Possible role of peptidergic fibres in the carotid labyrinth

In mammalian vascular systems, many physiological and pharmacological studies have suggested the vasoactive nature of SP, CGRP, VIP, and NPY, which are major neuropeptides in the carotid labyrinth, in relation to vascular smooth muscle cells, although SP and CGRP are originally involved in sensory mechanisms, and are putative sensory transmitters (see Iversen, 1982, for reviews). SP (Hallberg and Pernow, 1975; Samnegard et al., 1978; Edvinsson et al., 1981; Edvinsson and Uddman, 1982), CGRP (Brain et al., 1980), and VIP (Larsson et al., 1976; Heistad et al., 1980; Wilson et al., 1981) are thought to have a vasodilatory effect, and NPY (Lundberg et al., 1982; Edvinsson et al., 1983) is thought to have a vasoconstrictory one. In addition, FMRF causes dose-dependent contraction of the smooth muscle cells of the anterior aorta of the snail (Grifond et al., 1986), although the frequency of occurrence in the labyrinth is lower than that of the four peptides described above. Also, in the bullfrog dorsal aorta, iliac artery, and femoral artery CGRP has been shown to be a
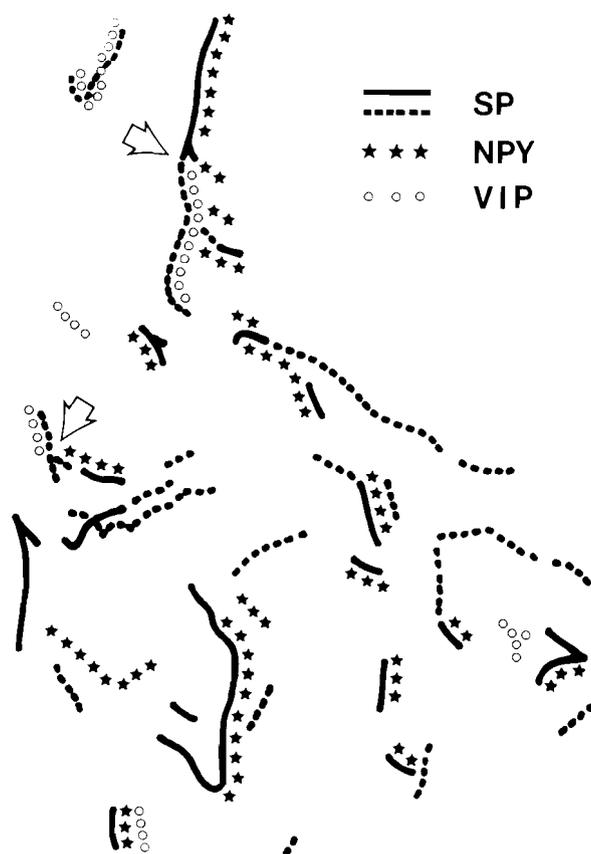

Fig. 21. Schematic illustration combining two serial sections (Figs. 19, 20). At two points (arrows), there are SP-immunoreactive fibres with VIP-immunoreactivity in one section, connected with SP-fibres with NPY-immunoreactivity in another section. (Kusakabe et al., 1994a. Cell Tissue Res. vol. 276. Springer-Verlag).

Table 3. The patterns (I-IX) of the possible coexistence of SP, CGRP, NPY, and VIP in the nerve fibres of the carotid labyrinth based on the present results by using these four peptide anti-sera. Filled circles (●) indicate the existence of immunoreactivity of these peptides, and open circles (○) indicate the absence of it. (Kusakabe et al., 1994a, Cell Tissue Res., Vol. 276, Springer-Verlag).

	SP	CGRP	NPY	VIP
I	●	●	●	●
II	●	●	●	○
III	●	●	○	●
IV	●	●	○	○
V	●	○	●	○
VI	●	○	○	●
VII	●	○	○	○
VIII	○	○	●	○
IX	○	○	○	●

vasodilator (Kline et al., 1988). Based on this, we have proposed that the target of these peptidergic fibres is the smooth muscle cells abundantly distributed in the intervascular stroma of the labyrinth, and that the peptidergic fibres are involved in vascular regulation in the carotid labyrinth (Kusakabe et al., 1991; Kusakabe, 1992c), although the possibility that these peptides also participate in the chemoreception in the carotid labyrinth cannot be ruled out. The similar peptidergic mechanism has been proposed in the amphibian kidney and lung (Kusakabe et al., 1994b,c). An immunohistochemical study at electron microscopic level (Matsuyama et al., 198e), which demonstrated the approach of SP immunoreactive terminals to the smooth muscle cells in the cerebral artery, supports our speculation.

In the mammalian carotid body, it has been considered that SP- and CGRP-immunoreactive fibres are involved in chemosensory mechanisms (Helke et al., 1980; Jacobowitz and Helke et al., 1980; Wharton et al., 1980; Lundberg and Hökfelt, 1983). In the avian carotid body, Kameda (1989) has suggested a vascular regulatory function as well as the chemosensory one as a possible role of SP, CGRP, and VIP, and this suggestion is supported by physiological experiments in the cat carotid body, which showed that SP led to a change in chemosensory discharge through its effect on blood flow (McQueen, 1980). In the mammalian and avian carotid bodies there are no smooth muscle cells, except for the periphery of small arteries and arterioles. In contrast, the amphibian carotid labyrinth has many smooth muscle cells in the intervascular stroma of the labyrinth as shown in Fig. 2. The difference in function may depend on this. Thus, it seems that the role of peptidergic fibres in arterial chemoreceptor organs varies among species.

In the case of the coexistence of SP and CGRP, it has been considered that co-release of two peptides would synergically effect vasodilation (Kusakabe et al., 1993b). Consequently, the direct regulation by the peptidergic fibres may be controlled in part by the interaction of these two peptides. As shown in Table 3, in the case of the coexistence of SP and VIP, and SP, CGRP, and VIP, similar controls are supposed, because these three peptides are vasodilators. On the other hand, in the case of coexistence with NPY, co-release of NPY may exert a negative feedback type of regulatory role because the effect of NPY is opposite to that of SP, CGRP, and VIP (Kusakabe et al., 1994a). In the case of the coexistence of three or four peptides (Table 3), a more complicated interaction is speculated. However, we do not yet have direct evidence for this.

Ishii and Ishii (1973) have physiologically confirmed the fibre composition and derivation of afferent and efferent nerve fibers in the carotid/sinus nerve of the toad. Most carotid nerves are derived from the sympathetic nerve, and partly from the vagal nerve. The sympathetic fibers are vasoconstrictors of the vasculature of the carotid labyrinth, and the vagal nerves are chemosensory and barosensory. At present, we consider that the regulation of vascular tone in the carotid labyrinth is controlled by three different mechanisms: 1) indirect regulation through the g-s connection; 2) direct regulation by the sympathetic nerves; and 3) direct regulation by the peptidergic fibers.

Finally, morphological similarity between the amphibian carotid labyrinth and the carotid body in chronically hypoxic rats has been suggested (Kusakabe et al., 1993b). When the rats are exposed to chronic hypoxia, the carotid bodies are enlarged. As a result of enlargement, the carotid body shows a spongy appearance in light microscopy with large sinusoidal spaces. In addition, the ultrastructural characteristics of the glomus cells in chronically hypoxic rats resemble those in the normal amphibian carotid labyrinth. In chronically hypoxic rats, the arterial O_2 tension decreases from 87 ± 3.1 to 41.8 ± 1.6 torr (Aaron and Powell, 1993). This value is similar to the arterial O_2 tension in undisturbed conscious toads, although that in the toads varies widely (West et al., 1987). This indicates that our previous studies on the amphibian carotid labyrinth are useful to clarify the complicated chemosensory mechanisms of the mammalian carotid body in the pathological condition of hypoxia.

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