**Distribution of FMRFamide-Immunoreactive Nerve Fibers in the Carotid Labyrinth of the Bullfrog, *Rana catesbeiana* in Corresponding Differential Interference-Contrast (Nomarski) Images**

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**Summary.** Immunoreactivities for FMRFamide and substance P (SP) in the carotid labyrinth of the bullfrog were detected using the peroxidase-antiperoxidase method, and the results compared with corresponding differential interference-contrast (Nomarski) images. Colocalization of both peptides was determined by the indirect double immunofluorescence method. Immunoreactivities for FMRFamide and SP were found in nerve fibers distributed in the intervascular stroma of the labyrinth. The FMRFamide-immunoreactive fibers were less numerous than the SP-immunoreactive fibers. In the Nomarski image, FMRFamide-fibers were recognized in relief, with most of them located near the walls of blood vessels. All FMRFamide-fibers coexisted with SP. The results suggest that FMRFamide-immunoreactive fibers are also involved in local vascular regulation of the carotid labyrinth.

The amphibian carotid labyrinth is a complex vascular expansion where the common carotid artery bifurcates into the internal and external carotid arteries (Adams, 1958). Ishii et al. (1966) demonstrated electrophysiologically in the toad that the carotid labyrinth is an arterial chemoreceptor corresponding to the mammalian carotid body and carotid sinus. On the other hand, Ishida (1954), Carman (1955), and Banister et al. (1975) previously suggested a vascular regulatory function for the labyrinth, though without direct evidence for this fact which we subsequently confirmed physiologically (Kusakabe et al., 1987).

Recently, Kusakabe et al. (1991) and Kusakabe (1992a) performed immunohistochemical studies demonstrating several neuropeptides including substance P (SP), calcitonin gene-related peptide (CGRP), and vasoactive intestinal polypeptide (VIP) in the nerve fibers within the adult and developing carotid labyrinths. As these peptides have been known to be vasoactive (Hallberg and Pernow, 1975; Samnegard et al., 1978; Heistad et al., 1980; Edvinsson et al., 1981; Wilson et al., 1981; Edvinsson and Uddman, 1982; Brain et al., 1985), the results suggest that they might be involved in the vascular regulation of the labyrinth in collaboration with direct regulation by the sympathetic system and an indirect system through the g-s connection (close apposition of the glomus and smooth muscle cells) (Ishii and Kusakabe, 1982; Kusakabe et al., 1987; Kusakabe, 1992b, c).

FMRFamide (Phe-Met-Arg-Phe-NH₂), a molluscan cardio-excitatory tetrapeptide which has been obtained from ganglia of the clam (Price and Greenberg, 1977), is also known to exert cardiovascular effects (Mues et al., 1982; Barnard and Dockray, 1984; Wong et al., 1985; Chai et al., 1986; Grifond et al., 1986; Roth et al., 1987). Immunohistochemical evidence of the occurrence of FMRFamide has been obtained in the nervous system in a variety of animal species: coelenterates (Grimmelikhuijzen et al., 1982; Grimmelikhuijzen, 1983), insects (Bore et al., 1980), molluscs (Martin et al., 1981), fishes (Bore et al., 1980; Jirikowski et al., 1984; Stell et al., 1984; Bonn and Konig, 1988, 1989a, b; Ohtomi et al., 1989; Ostholm et al., 1990; Chiba et al., 1991; Fuji and Kobayashi, 1992; Vecino and Ekström, 1992; Wicht and Northcutt, 1992), amphibia (Dockray et al., 1981), birds (Dockray et al., 1981; Wirsig-Wiechmann, 1990), and mammals (Bore...
et al., 1980; Dockray et al., 1981; Hökfelt et al., 1983; Williams and Dockray, 1983; Sasek and Elde, 1985; Wirsig and Basinger, 1988). There are, however, no immunohistochemical studies on the occurrence of FMRFamide in the amphibian carotid labyrinth. Information as to whether this substance is involved in the nerve fibers in the labyrinth would aid in understanding the peptidergic system in this organ.

In the present study, we examined FMRFamide-immunoreactivity in combination with differential interference-contrast (Nomarski) images. We also used double-immunostaining for FMRFamide and SP, which is abundantly distributed in the labyrinth (Kusakabe et al., 1991; Kusakabe, 1992a), to compare the distribution of these peptides.

**MATERIALS AND METHODS**

Seven bullfrogs, Rana catesbeiana, weighing 250–300 g, were used. The animals were pithed, and perfused intracardially with heparinized (1 IU/ml) 0.1 M phosphate buffer saline (PBS), pH 7.4, followed by 300 ml of Zamboni's fixative (Zamboni and DeMartino, 1967). Both carotid labyrinths from each were removed and immersed in the same fixative for an additional 6h at 4°C. After a brief washing with PBS, the specimens were transferred to 30% sucrose in PBS and kept there overnight at 4°C. The specimens were then cut serially at 20 μm on a cryostat, and mounted on gelatinized slides. These sections were

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**Fig. 1 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. Arrows indicate several fine fibers 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 fibers are distributed in the intervascular stroma. The immunoreactive fibers indicated by arrows correspond to the relief fibers in A. Many nerve fibers marked by arrowheads in A are not immunoreactive for FMRFamide in B. Scale bar=50 μm.
immunohistochemically stained according to the peroxidase-antiperoxidase (PAP) method for individual staining of FMRFamide (Incstar) and SP (Sera-lab) and by the indirect immunofluorescence method for double-staining. These processes of immunostaining have been detailed in our previous reports (KUSAKABE et al., 1991, 1993).

The sections immunostained by the PAP method were examined with an Axiomat microscope (Zeiss) equipped with a halogen lamp. From the same area where the immunoreactivity was observed, a differential interference-contrast (Nomarski) image was also taken with the same microscope to see the arrangement of the vascular maze. For a fluorescent image, an HBO 100 super pressure mercury lamp (Osram), an exciting filter system (546 nm for rhodamine, 450-490 nm for FITC), and an emission filter system (590 nm for rhodamine, 515-565 nm for FITC) were attached to the Axiomat microscope.

The reactions for either peptide were additionally verified by treating sections with the primary antibody, and incubating them overnight with 50 μM of either peptide. To test the specificity of the antisera, the sections treated with FMRFamide antibody were incubated with rhodamine-conjugated anti-rat IgG, and those treated with SP antibody were incubated with FITC-conjugated anti-rabbit IgG.

RESULTS

A Nomarski image of a 20 μm-thick cross section of the carotid labyrinth shows a three-dimensionally complicated vascular maze consisting of the sinusoidal plexus and intervascular stroma (Fig. 1A). The edge between the sectioned intervascular stroma and sloping surface of the endothelium is easily discriminated.

In sections stained by the PAP method, immunoreactivities for FMRFamide (Fig. 1B) and for SP were found in nerve fibers distributed in the intervascular stroma of the labyrinth. These fibers appeared as thin processes with several varicosities. The infrequent FMRFamide-immunoreactive fibers were localized in a restricted area of the labyrinth. SP-immunoreactive fibers were numerous and distributed throughout the entire labyrinth as previously reported by KUSAKABE et al. (1991, 1993). The FMRFamide-immunoreactive fibers tended to be distributed in the central portion rather than in the peripheral portion of the labyrinth. Thus, there were some distinct differences in the distribution and abundance between these two fibers. In the Nomarski image of the same area of the immunostained section, some fine fibers could be seen in relief (arrows), corresponding to the immunoreactive fibrils.

Fig. 2 A and B show the same region of a cryostat section of the carotid labyrinth. Double immunostaining for FMRFamide (A) and SP (B). FMRFamide-immunoreactive fibers are less numerous than SP-immunoreactive fibers. The arrow in A shows a fiber immunoreactive for both FMRFamide and SP. Scale bar = 50 μm.
fibers (Fig. 1A, B). All immunoreactive fibers had corresponding reliefs in the Nomarski image. Although most immunoreactive fibers in the immunostained sections seemed to run through the center of the intervascular stroma, in the Nomarski images it was clear that many of them really ran close to the blood vessels, because the area which seemed to be the intervascular stroma was actually a sloping section of an endothelium. On the other hand, in the Nomarski images of the sections immunostained for FMRFamide, there were also fibers in relief which did not correspond to the immunoreactivity (arrowheads in Fig. 1A, B). These may be SP-immunoreactive fibers.

The findings of the double-immunostaining of the two peptides agree well with the results obtained from individual immunostaining with the PAP method. Many fibers showed the fluorescence originating from rhodamine (SP-immunoreactive fibers) and a small number of fibers showed fluorescence from FITC (FMRFamide-immunoreactive fibers) (Fig. 2A, B). All fibers showing fluorescence from FITC also showed fluorescence from rhodamine.

No nerve fibers with the immunoreactivity for FMRFamide and SP were detected in sections of the carotid labyrinth incubated in the preabsorbed antisera. In addition, no nerve fibers with SP-immunoreactivity were found in sections incubated in FITC-conjugated anti-rabbit IgG, and no fibers with FMRFamide immunoreactivity were detected in those incubated in rhodamine-conjugated anti-rat IgG. Thus, we confirmed that these two primary antibodies represented specific differences, and did not show cross-reactivity with the second antibodies.

**DISCUSSION**

GRIFFOND et al. (1986) demonstrated immunoreactivity for FMRFamide in the nerve fibers supplying the smooth muscle cell layer of the anterior aorta of the snail, *Helix aspersa*. They also examined the effects of FMRFamide *in vitro*. A physiological dose (3 x 10^-7 M) of FMRFamide caused dose-dependent contractions of the smooth muscle cell layer. From these data, they proposed a mechanism for the action of FMRFamide through the smooth muscle cells on the cardiovascular system. Many physiological works have also suggested that SP, CGRP, and VIP have a vasodilator effect in the various vascular systems (HALLBERG and PERNOW, 1975; SAMNEGARD et al., 1978; HEISTAD et al., 1980; EDVINSSON et al., 1981; WILSON et al., 1981; EDVINSSON and UDDMAN, 1982; BRAIN et al., 1985). Based on this, we recently proposed that fibers containing these peptides are involved in vascular regulation in the labyrinth, and that the target of these fibers is the smooth muscle cells abundantly distributed in the subendothelial stroma (KUSAKABE et al., 1991, 1993; KUSAKABE, 1992a). SP-fibers may also be involved in sensory mechanisms, as has been proposed for mammalian chemoreceptor organs (HELKE et al., 1980; JACOBOWITZ and HELKE, 1980; WHALTON et al., 1980; LUNDBERG and HOKFELT, 1983). In this study, FMRFamide was demonstrated, for the first time, in the nerve fibers within the intervascular stroma of the labyrinth, although the frequency of occurrence was lower than that of SP-fibers. FMRFamide-fibers also may be involved in the vascular regulatory function of the labyrinth in addition to SP, CGRP, and VIP fibers, as previously suggested (KUSAKABE et al., 1991, 1993; KUSAKABE, 1992a).

All FMRFamide-fibers coexisted with SP-fibers. Recently, we reported the immunohistochemical co-existence of SP and CGRP in most nerve fibers distributed in the labyrinth of bullfrogs (KUSAKABE et al., 1993). Thus, the coexistence of three different peptides in a small number of nerve fibers in the labyrinth is probable. In the case of the co-release of SP and CGRP, we envision an interaction between these two peptides, because both SP and CGRP have a similar vasodilator effect (KUSAKABE et al., 1993). In the present case of coexistence, however, FMRFamide has a vasoconstrictor effect, and SP, a vasodilator one. That is, the mode of interaction of FMRFamide and SP may differ from that of SP and CGRP, because the effect of the present two peptides in the vascular system is just the opposite.

WONG et al. (1985) examined the effects of intracerebroventricular injection of FMRFamide on blood pressure in anesthetized rats. A small amount of FMRFamide (100 and 300 nM/rat) caused a rapid initial elevation of blood pressure and a great increase in pulse pressure, but these effects were abolished by pre-treatment with naloxone. On this basis, they concluded that FMRFamide may act via the naloxone-sensitive opiate receptors in the brain. CHAI et al. (1986) also provided evidence for the significance of FMRFamide in the central modulation of cardiovascular functions. In our Nomarski image, most FMRFamide-fibers were located near the walls of blood vessels. Previous ultrastructural studies showed that some attenuated segments of the endothelial cells displayed the fenestration usually found in the capillaries of the endocrine organs, and that some nerve terminals with numerous clear vesicles, 40 nm in diameter, directly contacted the surrounding subendothelial connective tissue without the covering of a supporting cell (KUSAKABE, 1990, 1991, 1992c). In light of the present and previous studies, FMRFamide may be released in the relatively limited area of subendothelial stroma. Consequently, the released FMRFamide may enter the vascular lumen through the fenestrat-
ed membrane and/or the attenuated segments of the endothelial cell. To confirm this speculation, further physiological experiments are required.

In conclusion, the present findings suggest that FMRFamide-immunoreactive fibers are also involved in the local vascular regulation of the labyrinth in addition to previously reported SP, CGRP, and VIP.

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REFERENCES


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