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Immunohistochemical Study of Vasoactive Intestinal Peptide(VIP) Containing Nerve Fivers in Encephalic Pial Arteries of the Horse Shoe Bat

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ABSTRACT : The distribution of nerve fivers containing vasoactive intestinal peptide (VIP) in small pial arteries of the horse shoe bat was investigated by means of immunohistochemistry. Strongly labeled VIP-immunoreactive (VIP-IR) fiber bundles were observed in encephalic pial arteries and gave rise to small varicose fibers organized in a meshwork pattern of the nerve fibers. The highest density of meshwork pattern of VIP-IR fibers was observed in the large diameter blood vessel (about 200 μm in diameter) in present study. On the small pial arteries (about 50 μm in diameter) and arteriole (diameter$\leqq$30 μm), VIP-IR fiber appeared to be a single fiber or non-fiber. It showed from the preparation of cross section in blood vessels that the VIP innervation of the pial arteries was mainly located at the adventitio-medial junction. It is interesting that the vasa vasorum vascularizing tunica adventitia and external part of tunica media of large or middle arteries included a thin single VIP-IR nerve fiber.

Introduction

Vasoactive intestinal peptide (VIP) was originally believed to be solely a gut hormone since its discovery in V. Mutt's laboratory in Stockholm (Sweden) from one of the peptide fractions which remained after the purification of secretin extracted from hog upper intestinal tissue[1,2]. Thereafter, VIP was found in detectable concentrations in neuroblastoma and astrocytoma cell lines as well as in the dog brain tissue[3]. This was the starting point for a great number of data suggesting the existence of the same peptide both in the central and peripheral nervous-system. Recently, VIP was immunohistochemically localized in various regions of the central and peripheral nervous system.

The localization by immunohistochemical techniques of VIP nerves innervating cerebral blood vessels in the cat was the morphological support for the hypothesis suggesting that VIP may play a role in the regulation of cerebral blood flow[4]. Indication for the role of VIP in central blood flow regulation is given by both *in vivo*[5-8] and *in vitro*[4,9,10] studies.

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Materials and Methods

Twelve adult horse shoe bats (*Rhinolophs ferrumequinum*), with body weights 19.5-23.5 g, were used in this study. All bats were collected from Monzen, Oga peninsula, Akita prefecture, Japan between October 1992 to December 1995. The animals undergoing surgery were deeply anesthetized with sodium pentobarbital (50 mg/kg;i.p.) and perfused through the ascending aorta first with 10 ml washout solution (0.1M phosphate buffer, pH 7.4, 0.025% heparine) and 100 ml of fixative solution (4.0 % paraformaldehyde and 0.2 % picric acid in 0.1 M phosphate buffer, pH7.4). After perfusion, the brain with pial arteries was removed and immersed in same fixative solution for 1 day[11]. The brain with pial arteies was cut into 50 μm serial sections on a Vibratome (Oxford) and stored in the 10 mM phosphate buffer saline (PBS) that contains a 0.1 % Triton-X 100. The visurization procedure was performed on free-floating sections[12-14]. The sections were rinsed in PBS and placed in 0.3 % H_2O_2 for 30 minutes to eliminate endogenous peroxidase activity. After 3 rinses (10 minutes each) in PBS, the sections were incubated with anti-vasoactive intestinal peptide(VIP) antibody (Eugen Tech, U.S.A) in PBS (pH 7.4;dilution rate 1:2,000) for 3 days at 4℃. The sections were then rinsed 3 times in PBS, the sections were incubated with goat anti-rabbit IgG (Miles, U.S.A.) in PBS (dilution rate 1:200) for 2 hours at room temperature, and incubated with a rabbit PAP complex (Dakopatts, Denmark) in PBS (dilution rate 1:200) for 2 hours at room temperature. The sections were then rinsed 3 times in 50 mM Tris-hydrochlonic acid buffer for 10 minutes each. The sections underwent a reaction in 0.05 % diaminobenzidine (Sigma, U.S.A) in 0.01 % H_2O_2 in 50 mM Tris HCl buffer (pH. 7.5) to develop the color for 7 minutes at room temperature[15]. The sections were mounted on aminoalkylsilane-treated slides[16]. After drying into 37℃ incubator for 1 day, the mounted sections were defatted in two changes of 50% chloroform:50% ethanol for 2 hours each, and rehydrated through an ethanol series. Then these placed in 0.005% solution of osmium tetroxide in distilled water for 30 minutes. Following a 15 minutes rinse in running distilled water, the sections were immersed in 0.05% solution of thiocarbohydrazide in distilled water for 30 minutes and then sections were rinsed in distilled water for 15 minutes, dehydrated a graded series of ethanol, and coverslipped[17]. The preparations were observed using an optic microscope and representative sections were photographed with Zeiss Axiophoto microscope.

Results

The diameter of small pial arteries was observed in various sizes (about 200 μm～10 μm). The pattern of vasoactive intestinal peptide (VIP)-immunoreactive (-IR) nerve plexuses on the incepharic pial arteries of the horse shoe bats demonstrated changes with diameter of arteries.

In the large sized arteries (about 200 μm in diameter:Figs.1-5) on this investigation, approximately similar pattern of distribution was found in every artery examined on this study. VIP-IR fivers run irregularly along the arterial walls, and they sometimes form fiber bundles (Figs.1-3;large arrow head). From these bundles consisting of two to three thick axons, thin fibers were ramified repeatedly and joined with the formation of meshwork patterns (Figs.1-3). Strongly labeled VIP-IR fiber bundles were given rise to small varicose fibers organized in a meshwork pattern (Figs.1-3: small arrow heads). VIR-IR long longitudinally oriented bundles in a meshwork pattern were found in some large sized arteries (Fig.2). Numerous mostly circumferentially oriented, bead like varicosities were occupied the greater part of these large sized arteries (Fig.3). These circumferentially oriented fibers

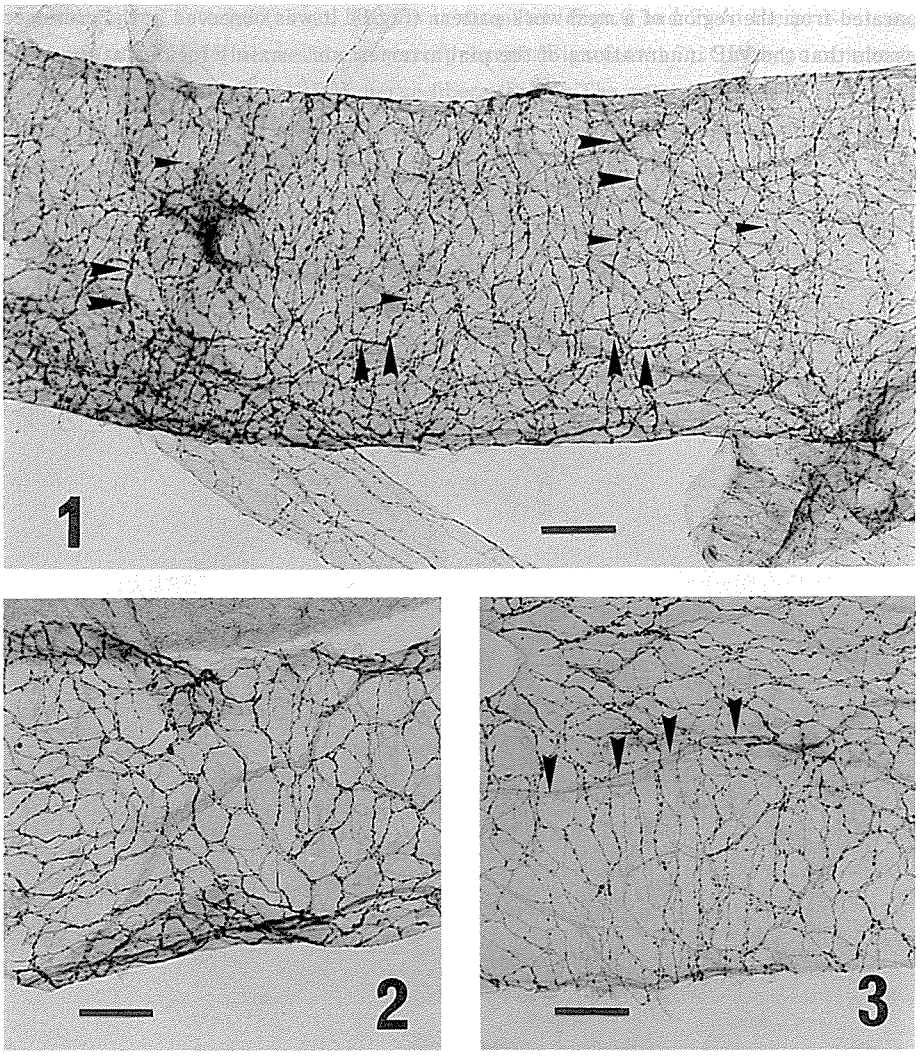


Fig.1. Photomicrograph illustrating vasoactive intestinal peptide (VIP)-immunoreactive (IR) nerve plexus around the large pial artery (about 200 μm in diameter). Major fiber bundles (large arrow heads) and small varicosities (small arrow heads) are indicated. VIP-IR branched fibers consist of a meshwork pattern and a circumferential pattern. Scale bar, 50 μm.

Fig.2. Photomicrograph illustrating VIP-IR nerve plexus around the large pial artery (about 200 μm in diameter). This plexus showing a typical meshwork pattern of the nerve fibers. Scale bar, 50 μm.

Fig.3. Photomicrograph illustrating VIP-IR nerve fibers around the large pial artery (about 200 μm in diameter). The nerve fibers surrounding blood vessel giving a circumferential pattern. A VIP-IR single nerve fiber (arrow heads) showing into the vasa vasorum vascularizing tunica adoventitia and the external part of tunica media of artery. Scale bar, 50 μm.

were separated from the region of a meshwork pattern (Fig.1). It was observed at the cross sections of blood vessels that the VIP innervations of the pial arteries were mainly located at the adoventitio-medial junction (Figs.4 and 5).

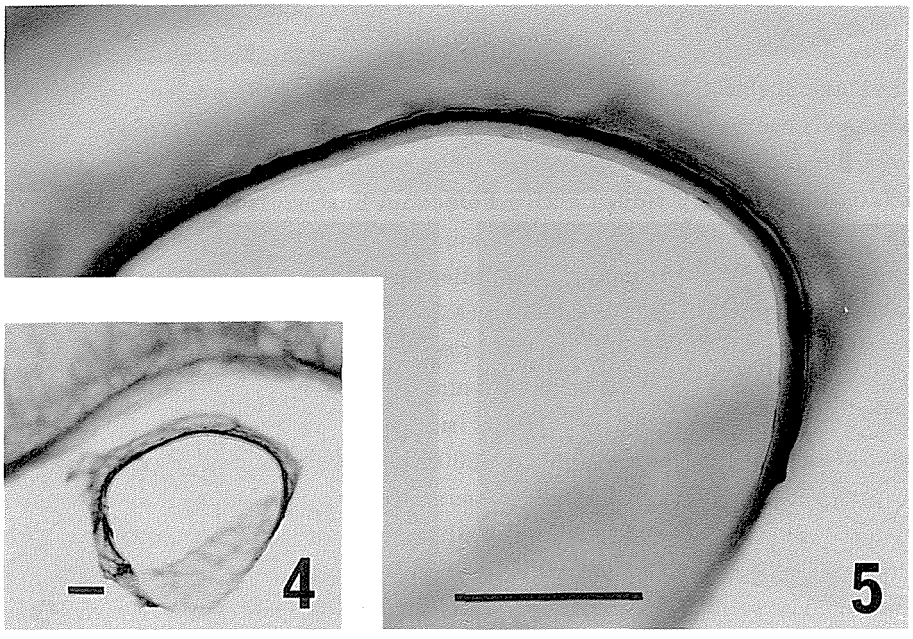
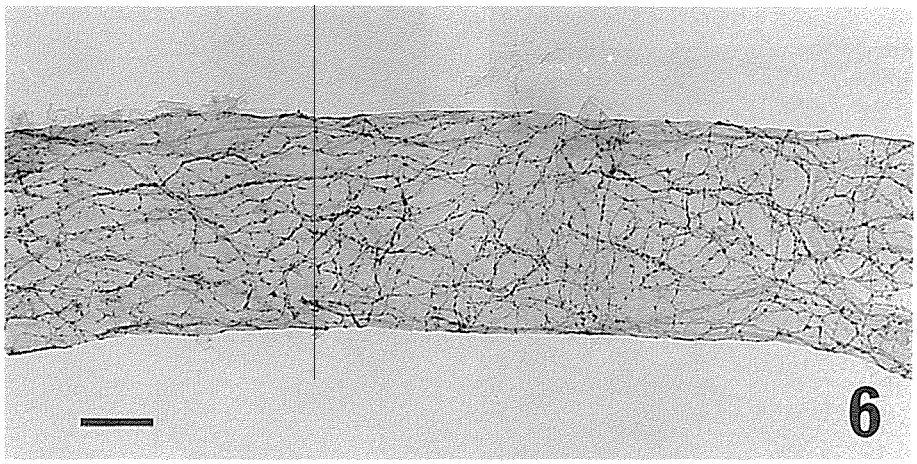


Fig.4.
Photomicrograph illustrating the cross section of pial arteries with VIP-IR nerve fibers. VIP-IR nerve fibers locate at the adoventitio-medial junction. Scale bar, 50 μm.

Fig.5.
Showing the enlargement of the Fig.4. Scale bar, 50 μm.

In the middle sized arteries (about 100 μm in diameter: Figs.6 and 7) on the present study, the two type patterns of VIP-IR nerve fibers were present (Figs.6 and 7). One type of arteries was arranged in a meshwork pattern of VIP-IR nerve fibers (Fig.6). The another type was mainly consisted of VIP-IR long longitudinal nerve fibers and the oblique nerve fibers participated in these fibers (Fig.7). First type was observed predominantly in these middle sized arteries.



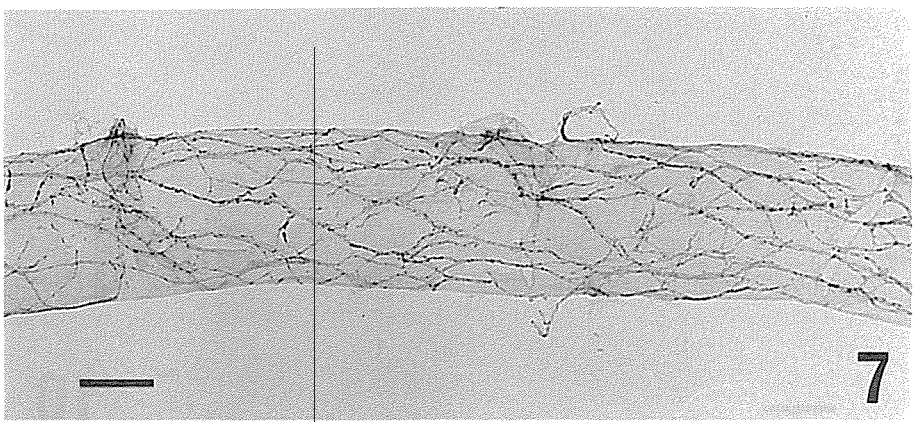


Fig.6.
Photomicrograph illustrating VIP-IR nerve fibers around the middle pial artery (about 100 μm in diameter). These fibers showing a fine meshwork pattern. Scale bar, 50 μm.

Fig.7.
Photomicrograph illustrating VIP-IR nerve fibers around the middle pial artery (about 100 μm in diameter). These fibers consist of long longitudinal and oblique nerve fibers. Scale bar, 50 μm.

In the small sized arteries (about 50 μm in diameter: Figs.8-15), the VIP-IR nerve fibers were showed mainly a few long longitudinal fibers, and circumferential or oblique fibers consisted of over a vessel wall (Figs.8 and 9). In these small sized arteries, thick VIP-IR fiber bundles were also observed (Figs 8 and 9, arrow head). The longitudinal and oblique fibers with small vaicosities were located over an artery wall. The distribution of VIP-IR fibers in ramification vesses decreased or got lost (Figs.10-12).

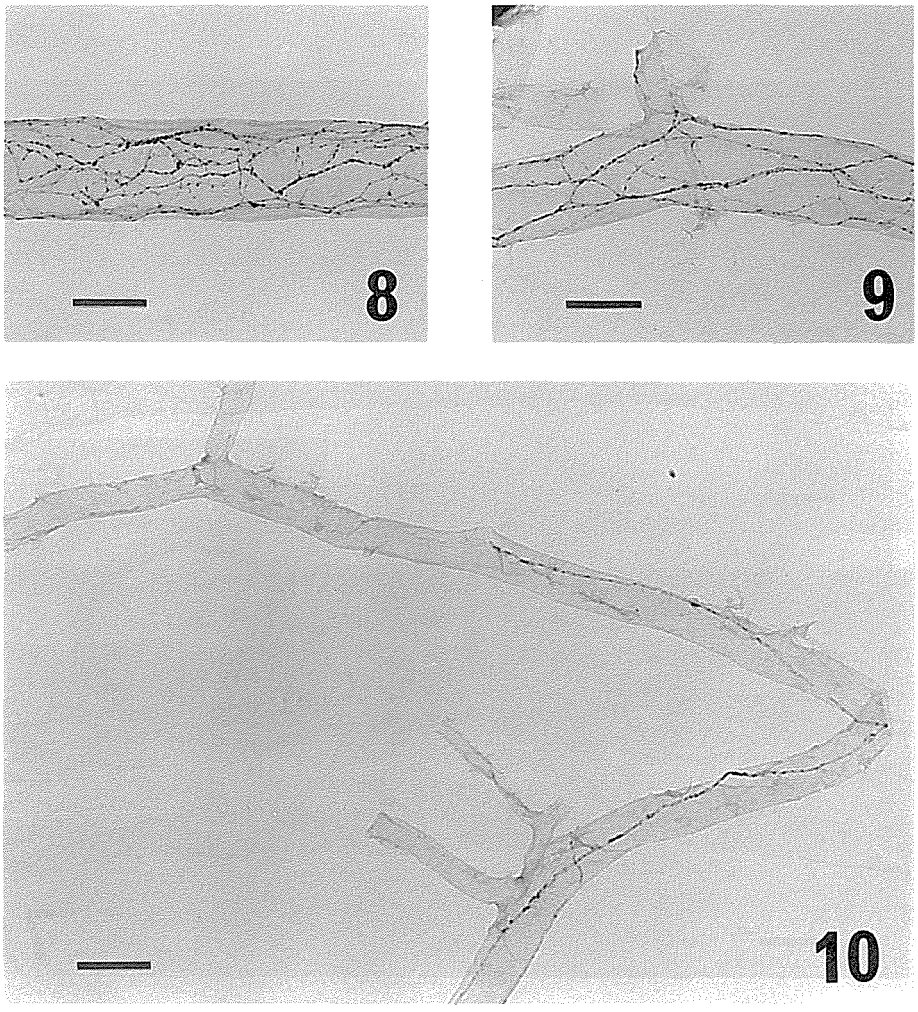
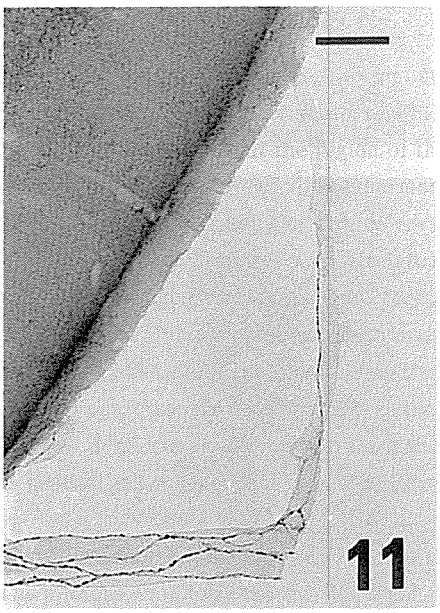


Fig.8. Photomicrograph illustrating VIP-IR nerve plexus around small pial arteries (about 50 μm in diameter). These fibers showing a fine meshwork pattern. Scale bar, 50 μm.

Fig.9. Photomicrograph illustrating VIP-IR nerve fivers consisted of longitudinal and oblique fibers around small pial artery (about 50 μm in diameter). Scale bar, 50 μm.

Fig.10. Photomicrograph illustrating the VIP-IR nerve plexus consisted of a single or a few fibers around small pial artery (about 50 μm in diameter) and branched arterioles (about 10 μm in diameter). The extensional region in the small artery is devoid of a VIP-IR nerve fiber. Scale bar, 50 μm.

In the arterioles (diameter≦30 μm: Figs.11-14), the VIP-IR nerve fibers located a single or a few longitudinal fibers and showed sometimes a few oblique fibers (Figs.11 and 13). Furthermore, VIP-IR fibers were not observed the extensional region in the arterioles or branched smaller arterioles (Figs. 10-12 and 14).



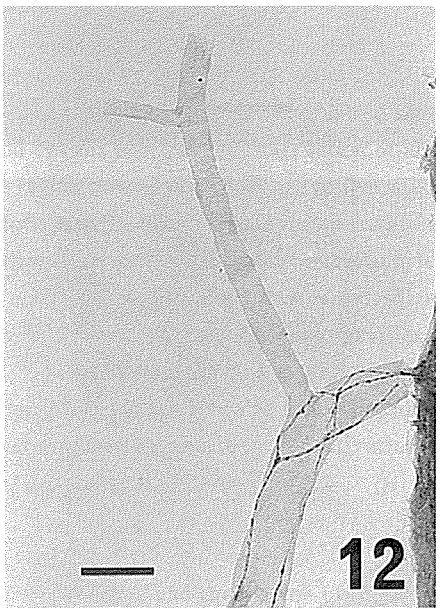
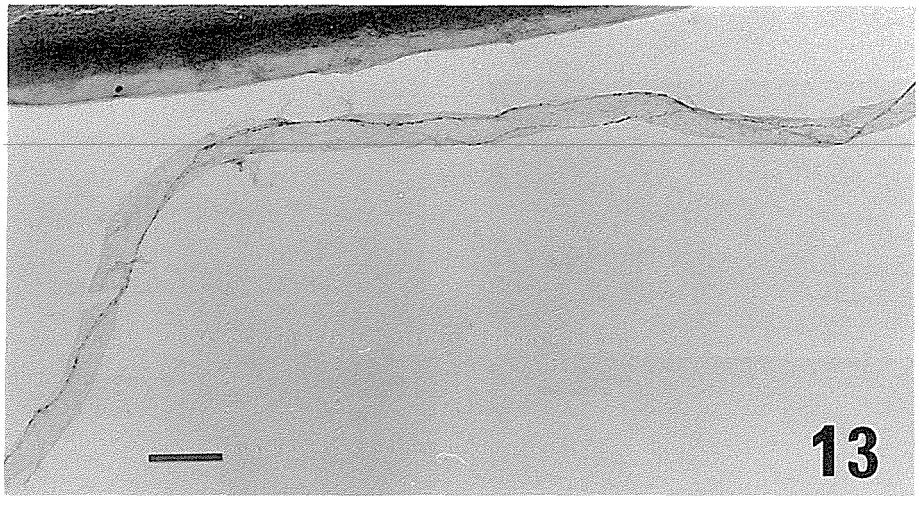


Fig.11.
Photomicrograph illustrating VIP-IR nerve fibers ran along small pial arteries (about 50 μm) and branched arteriole(about 10 μm in diameter) included VIP-IR nerve fibers. Scale bar, 50 μm.

Fig.12.
Photomicrograph illustrating VIP-IR nerve fibers ran along small pial artery (about 40 μm in diameter) and branched arterioles (about 10 μm in diameter) showed that excluding nerve fibers. Scale bar, 50 μm.



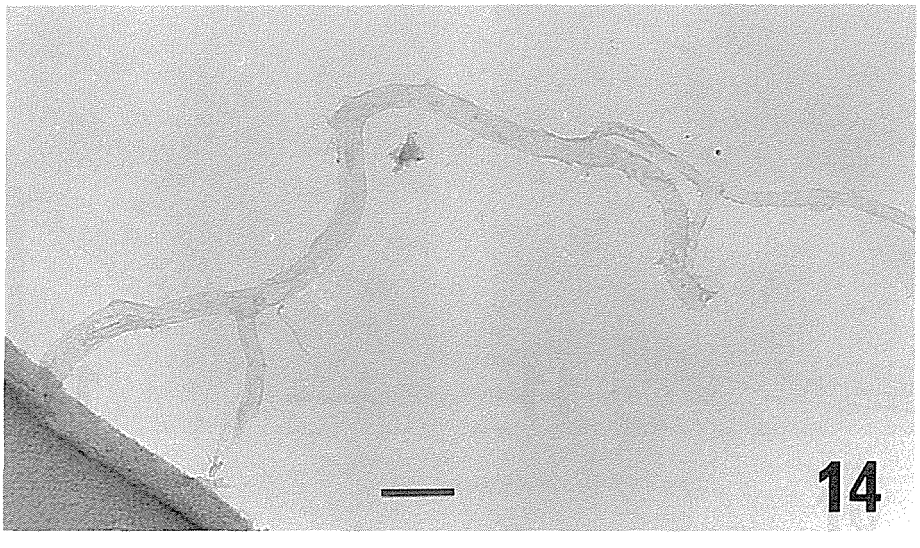


Fig.13.

Photomicrograph illustrating VIP-IR longitudinal nerve fibers ran along pial arterioles (diameter≦30 μm). All arterioles include nerve fibers. Scale bar, 50 μm.

Fig.14.

Photomicrograph illustrating branched pial arterioles (diameter≦30 μm). It is showed that branched arterioles excluded VIP-IR nerve fibers. Scale bar, 50 μm.

Discussion

The results of the present investigation have shown that small pial arteries of the horse shoe bat (*Rhinolophs ferrumequinum*) are richly supplied by vasoactive intestinal peptidergic (VIP) nerve fibers. This finding is based on VIP immunohistochemical staining procedure. In the previous study[18], sympathetic and other peptidergic innervations were demonstrated the pial arteries and its branches. The consistency of innervation revealed in this study shows the role of the VIP nervous system in the regulation of pial blood flow[4].

While VIP-immunoreactive (IR) fivers were seen in each of the various sized arteries without some arteriole, and the greatest VIP-IR density of fibers was observed in the thickest artery in the present study. The distribution pattern of VIP-IR nerve fiver differed somewhat depending on the same sized arteries or the region of the same arteries.

The density of VIP nerves innervating blood vessels in the central nervous system varies between species, high one is in pig, cat and rat, and low one is in monkey, mouse and rabbit[10,19]. Strongly labeled VIP-IR fibers were observed in various sized pial arteries of the bat and these were made a meshwork pattern. The present study of the bat suggested that the VIP innervation of small pial arteries was a strong density group in numerous species.

Some small arteries (diameter$\leqq$30 μm) and arteriole were sometimes devoid of the VIP-IR nerve fiber as sympathetic noradrenergic nerve fibers in encephalic pial arteries of the horse shoe bat[18]. In such cases, it is supposed that the region of the blood vessels receives the innervation of another peptide or some neurotransmitter[20-22]. Furthermore, the region devoid of VIP-IR nerve fivers is regulated by the VIP-IR nerve fibers on the wall of vessel before ramification. These hypotheses are supported by the report that VIP produces a dose related relaxing effect on the cat middle cerebral artery[4,10] and on the bovine anterior choroidal artery[9], furthermore, the increase in blood flow is accompanied by an increase in cerebral oxygen consumption and changes in electroencephalographic profiles in the baboon following opening of the blood-brain barrier[6]. While pial arteries are innervated by numerous peptides and neurotransmitters[20-25]. Especially, a recent investigation revealed that VIP stimulates cyclase activity in microvessels isolated from the rat cerebral cortex and that this activation is specific for the peptide and independent of catecholamine and prostaglandin effects[26].

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