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Autocrine endothelial regulation in brain stem vessels of newborn piglets

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Summary. Vasoactive intestinal peptide (VIP) is known as a potent regulator for the development of the central nervous system (CNS). The neonatal period of brain development is characterised by rapid cellular proliferation in parallel with neuronal differentiation and angiogenesis. We examined the expression of native VIP and the VIP receptor-associated protein by immunohistochemistry as well as the expression of VIP mRNA by *in situ* hybridisation in the brain stem of newborn piglets. We found both the mRNA and the protein of VIP as well as the VIP receptor-associated protein in endothelial cells of veins, arteries and capillaries in the marginal zone of brain stem tissue sections, especially in pons and mesencephalon, as well as in pial vessels. The coexpression of native VIP, VIP mRNA and the VIP receptor-associated protein within the endothelium suggests the presence of an autocrine loop, which has been detected so far only in neuroblastoma cells. This expression pattern gives evidence to the immaturity of endothelial cells at birth and the presence of an adaptive response in the VIP-regulated system during the change from intra- to extrauterine life.

Key words: Vasoactive intestinal peptide, VIP mRNA, VIP receptor-associated protein, Autocrine regulation, Endothelium, Brain stem

Introduction

Vasoactive intestinal peptide (VIP) is a member of the secretin/glucagon peptide family. It is a 28 amino acid peptide with a molecular weight of 3326 Da (Bodanszky et al., 1974; Said, 1984) and has been found in cholinergic and peptidergic neurons of the peripheral and central nervous system. VIP is produced as a precursor protein, prepro-VIP, which is then cleaved to VIP. The prepro-VIP contains other biologically active peptides such as the peptide with terminal methioninamide (PHM) and the peptide with terminal isoleucinamide (PHI), respectively (Itoh et al., 1983). These peptides are responsible for vasodilatation, relaxation of smooth muscles and stimulation of glandular secretion in various organs (Fahrenkrug, 1989).

In the central nervous system VIP acts as neurotransmitter and neuromodulator. Recent work has also suggested an important role in neuronal development. In vivo, VIP stimulates mitogenesis, induces secretion of trophic factors and supports neuronal survival (Brenneman et al., 1987, 1990). During the embryonic and fetal period, maternal VIP seems to support embryonic development by stimulating growth and differentiation. These effects are mediated by G-protein linked VIP-receptors which are already expressed prenatally (Gilman, 1984; Hill et al., 1994).

The occurrence of VIP in the perivascular plexus of cerebral vessels acting as a vasodilator is well known (Larsson et al., 1976; Sims et al., 1980; Tsai et al., 1992; Dauphin and Mackenzie, 1995; Zhu et al., 1997; Paspalas and Papadoupoulos, 1998). Perivascular VIP and noradrenaline-containing fibres have been localized in close proximity suggesting a combined action of both as vasodilatators in cerebral vessels (Zhu et al., 1995; Paspalas and Papadoupoulos, 1998). VIP receptors have been found in vascular smooth muscle cells (Lee and Saito, 1984; Poulin et al., 1986; Sidawy et al., 1989; Miao and Lee, 1991) but also at the luminal surface of cerebral vessels (O'Dorisio, 1987) and in cultured endothelial cells (Pasyk et al., 1992). Endothelium-dependent (Duckles and Said, 1982; Lee and Saito, 1984; Edvinsson et al., 1985) as well as endothelium-independent (Lee and Saito, 1984; Miao and Lee, 1991) VIP-induced vasodilatations have been described.

Human umbilical vessels express VIP (Cai et al., 1993). However, VIP has not been found in adult vascular endothelium (Chedotal et al., 1994). The precise function, significance and duration of the endothelial VIP expression is still not well understood.

The aim of this study was to investigate the role of VIP in the cerebral brain stem vasculature during very early postpartal adaptation. Therefore we examined the expression of VIP, VIP mRNA and the VIP receptor-

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associated protein in brain stem vessels of newborn piglets.

Materials and methods

Tissue

Brains of fourteen 24-48 hours old piglets (race Deutsches Land-/Edelschwein) were obtained after *in situ* perfusion fixation with 4% glutaraldehyde in phosphate-buffered saline (PBS), pH 7.4. The brain stem was divided into medulla oblongata, pons, mesencephalon and diencephalon. After rinsing in sucrose (10%, 15% and 20% in PBS) for 24 hours the tissue was frozen in chilled isopentane in liquid nitrogen.

The brain stem was divided into 10 parts as follows: 1, medulla oblongata; 2, caudal pons; 3, central pons; 4, rostral pons; 5, caudal mesencephalon; 6, central mesencephalon; 7, rostral mesencephalon; 8, caudal diencephalon; 9, central diencephalon; 10, rostral diencephalon .

In situ hybridisation

The different parts of tissue were sectioned (10-15 mm) and placed on slides pretreated with gamma-aminopropyltriethoxy-silane (Sigma, St. Louis, MO, USA). After fixation with 4% paraformaldehyde in PBS the sections were dehydrated and stored at -70 °C .

The 380 bp VIP/PHI cDNA (Donaldson et al., 1992) was inserted into the pGEM vector (Promega) and was linearized with NdeI. The antisense RNA probe was transcribed in vitro by the Sp6 RNA polymerase and labelled with [^{35}S]-UTP. After rehydration and pretreatment with proteinase K the sections were rinsed in 0.2% glycine (w/v) and acetylated in 0.1M triethanolamine, pH 8.0, 0.25% (v/v) acetic anhydride. Before applying the probe, the slides were rinsed in 50% formamide-2x standard saline citrate (SSC) at hybridisation temperature, dehydrated and dried. About 2x10^6 cpm/ml of ^{35}S-labelled RNA probe was applied onto the section in 2x SSC, 50% formamide, 10% (w/v) dextran, 1 mg/ml of tRNA, 1 mg/ml sheared herring sperm DNA, 10 mM dithiothreitol and 2 mg/ml bovine serum albumin (BSA) and hybridised in a humidified chamber overnight at a temperature of 55 °C. After washing with 50% formamide-2xSSC the slides were treated with RNase and rinsed in 2xSSC. Finally the slides were dehydrated and autoradiographed by using RPN40 hypercoat emulsion (Amersham) and exposed for 3 weeks in darkness at 4 °C. The tissues were counterstained with Mayer's hematoxylin and mounted. The control was performed with the same procedure using an irrelevant probe expressing only the vector sequence.

Immunohistochemistry

For immunohistochemistry we performed the same tissue preparation, but without perfusion. After rehydration the endogenous peroxidase was blocked with 0.3% H_2O_2. After rinsing in PBS containing 10% goat serum, 2% rat serum and 1% BSA the slides were incubated with the primary antibody overnight at 4 °C. We used a rabbit polyclonal anti-VIP-antibody (IgG, MILAB, Malmö, Sweden) diluted 1:5000 in PBS containing 0.1% BSA and a rat monoclonal anti-VIP receptor-associated protein-antibody (IgG2c, Immunotech S.A., Marseille, France) at a dilution of 1:50 in PBS containing 0.1% BSA. After rinsing in PBS the slides were incubated with the secondary antibody (biotinylated anti-rabbit IgG or anti-rat IgG, respectively) for 45 min at room temperature. The slides were incubated with ABC Elite Complex (Vector Laboratories, Burlingame, CA, USA) for 45 min at room temperature and eventually they were developed with 3-amino-ethylcarbazole in acetate buffer for 5-10 min. The counterstaining was done with Mayer's hematoxylin. For negative control primary antibodies were substituted with PBS containing 10% BSA.

Results

VIP mRNA expression

We found autoradiographic signals in the endothelium lining along the vascular spaces of arteries, veins and capillaries in the brain stem. Nearly all of the pial vessels showed positive signals detected as silver grains (Fig. 1). The cerebral vessels showing positive signals were localised mainly in the marginal region of the brain stem section. The highest density of reactive vessels were found in pons and mesencephalon. Only a few intracerebral vessels were labelled in the medulla oblongata and diencephalon.

Immunohistochemical expression of VIP

Homogenous, cytoplasmic labelling of endothelial cells was visible regardless of the type of vessel (Fig. 2). In large vessels there were immunoreactions also in the adventitia and the junction between media and adventitia. In small vessels only endothelial cells stained positive. The highly vascularised pia mater and the vessels in the marginal region of the brain stem sections stained strongly positive. The highest density of VIP-expressing vessels was detected in pons and mesencephalon.

Expression of VIP-receptor associated protein

We found a granular cytoplasmic pattern of immunostaining in the endothelium of arterial, venous and capillary vessels. Not the entire circumference of the vascular endothelial layer, but the majority of endothelial cells stained positive (Fig. 3). Positive reactions were also found in the adventitia and the junction between media and adventitia. All the pial

vessels were immunoreactive for the VIP receptor-associated protein. A similar staining pattern as for native VIP was visible intracerebrally, i.e. the immunoreactive vessels were located mainly in the marginal region of the brain stem section. The highest density of immunostained vessels was located in pons and mesencephalon.

Discussion

The aim of our study was to investigate the distribution of VIP and the VIP receptor-associated protein in brain stem vessels and thereby collect information about the possible role of VIP in the response of endothelial cells during the change from intra- to extrauterine life.

Concerning the cerebral vasculature it is worth recalling that the fetal brain of piglets is characterised by numerous leptomeningeal anastomoses between the anterior, middle and posterior cerebral arteries (De Reuck et al., 1972). These anastomoses are important resistance vessels for cerebral circulation and by this way they are involved in the regulation of cerebral blood flow (Armstead et al., 1989). The regression of the anastomoses has not finished completely at birth, indicating that important development and maturation processes of the cerebral vasculature continue after birth.

VIP, acting as a vasodilatator, plays an important role in blood flow regulation. Especially in the brain this mechanism has been investigated very extensively due to its importance for oxygen supply. It is also known that VIP is a highly potent factor during development of the nervous system as for neuronal growth and differentiation, axonal and dendritic development, synaptogenesis, and the development of neurotransmitter phenotypes, as well as neuronal death and gliogenesis. It

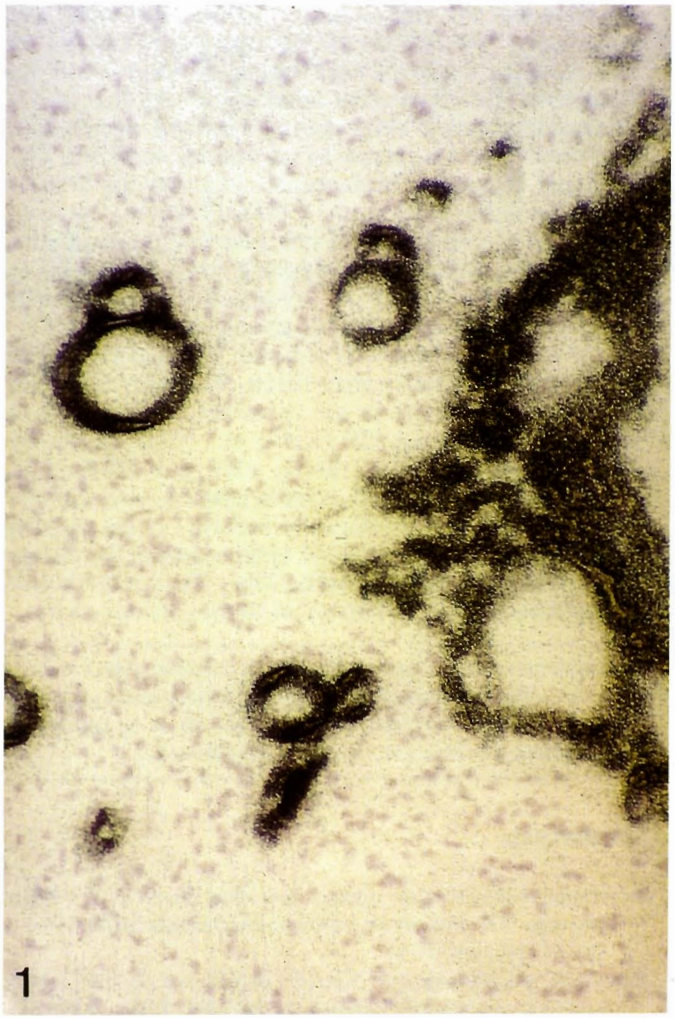

Fig. 1. Autoradiographic signals of VIP-mRNA in the endothelium of cerebral and pial vessels (diencephalon). x 100

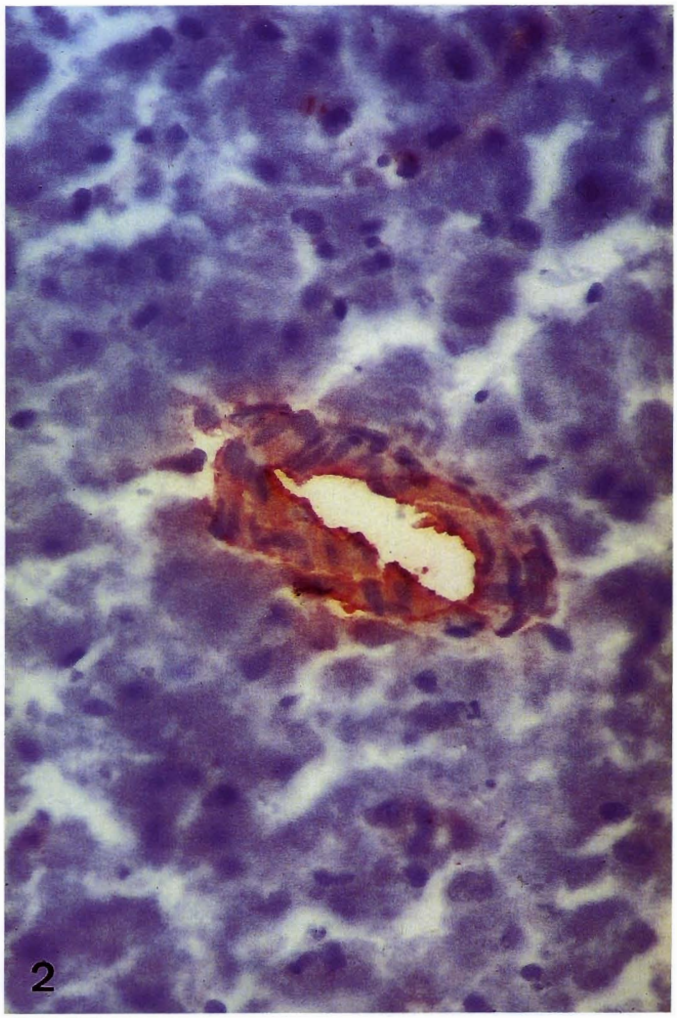

Fig. 2. Homogenous cytoplasmic VIP labeling of the vascular endothelium (diencephalon). x 250

has been demonstrated that endothelial cells of umbilical but not adult vessels express VIP (Cai et al., 1993). Therefore, we wanted to confirm whether there was a role of VIP in endothelial cells of cerebral vessels.

Since vascular development continues in the neonatal period we examined the brain stem vasculature of newborn piglets. The brain stem, a very dense organised region, contains numerous vital structures and specific neuronal nuclei. Considering their vital functions the regulation of cerebral blood flow has to be finely tuned under physiological and pathophysiological conditions. Furthermore, the vascular anatomy of the brain stem is fairly simple, allowing its precise examination.

We have studied piglets aged 24-48 hours when postnatal adaption has been initiated. Brain stem endothelial cells of piglets have shown positive signals for native VIP, VIP mRNA and the VIP receptor-associated protein, whereas the endothelium of adult animals does not express VIP mRNA or native VIP but the VIP receptor. The presence of native VIP, VIP mRNA and VIP receptor in the same cell, in this case the endothelial cell, is suggestive for the presence of an autocrine loop.

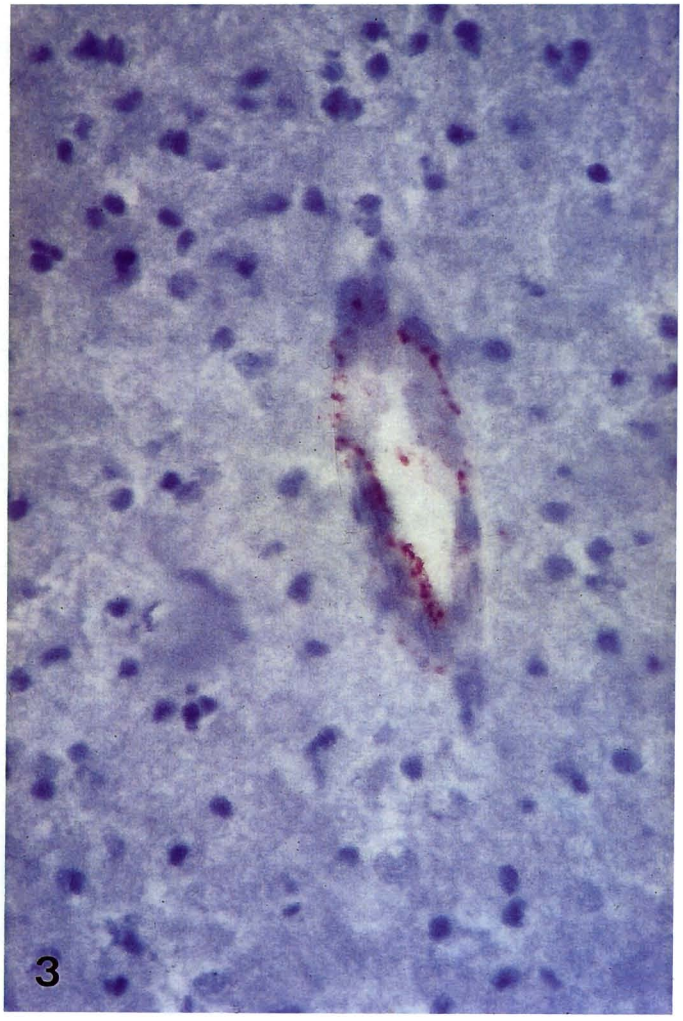

Fig. 3. Granular cytoplasmic pattern of immunostainings in vascular endothelial cells for the VIP receptor-associated protein (pons). x 250

An example of autocrine VIP-mediated growth regulation has been discovered in neuroblastoma cells (Muller et al., 1989; Waschek et al., 1989; Pence and Shorter, 1993; Wollmann et al., 1993). In this *in vitro* model VIP acts initially as a mitogenic factor. After numerous passages the cells develop to a distinct phenotype in which VIP mRNA and VIP immunoreactivity in the cells are lost, in association with the increase of VIP receptor expression. It is thus possible that the continuous presence of endogenous VIP during earlier passages modifies the number of VIP receptors and their responsiveness to VIP, which is accompanied by cell maturation and differentiation (Muller et al., 1989; Pence and Shorter, 1993).

The initial strong expression of VIP and its mRNA in the endothelial cells and also in neuroblastoma cells suggests that the expression of VIP is associated with immaturity. VIP and its mRNA expression but not the VIP receptor might be lost during differentiation and maturation. Based on other observations the loss of VIP expression seems to occur during a limited, not yet fully identified postnatal period. In rat pups an autocrine VIP-mediated regulation in neuronal cells was found at 14 days after birth (Hill et al., 1994). However, the exact time point of termination of this autocrine regulation has not yet been evaluated. Concluding from our results we assume the role of VIP as a differentiating and growth factor not only in neuronal but also in endothelial cells.

In contrast to the VIP receptor VIP and its mRNA are not expressed before birth. Prenatal VIP-VIP receptor interactions in the fetus are supposed to be mediated by maternal VIP (Hill et al., 1994). Due to the onset of VIP expression shortly after birth (Fink et al., 1987) and its limitation to a distinct period, as mentioned above, this mechanism might be involved in the adaptive response of the VIP-regulated system during the change from intra- to extrauterine life. To confirm this hypothesis, further physiological studies are necessary.

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