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Original article

Expression of VPAC1 receptor at the level of mRNA and protein in the porcine female reproductive system

R. Bukowski[1] , K. Wąsowicz[2]

[1] Department of Animal Anatomy, Faculty of Veterinary Medicine, University of Warmia and Mazury, Oczapowskiego 13, 10-957 Olsztyn, Poland
[2] Department of Pathophysiology, Forensic Veterinary Medicine and Administration, Faculty of Veterinary Medicine, University of Warmia and Mazury, Oczapowskiego 13, 10-957 Olsztyn, Poland

Abstract

The presence and distribution of vasoactive intestinal polypeptide (VIP) receptor VPAC1 was studied in the ovary, oviduct and uterus (uterine horn and cervix) of the domestic pig using methods of molecular biology (RT-PCR and immunoblot) and immunohistochemistry.

The expression of VPAC1 receptor at mRNA level was confirmed with RT-PCR in all the studied parts of the porcine female reproductive system by the presence of 525 bp PCR product and at the level of proteins by the detection of 46 kDa protein band in immunoblot. Immunohistochemical stainings revealed the cellular distribution of VPAC1 receptor protein. In the ovary it was present in the wall of arterial blood vessels, as well as in the ovarian follicles of different stages. In the tubular organs the VPAC1 receptor immunohistochemical stainings were observed in the wall of the arterial blood vessels, in the muscular membrane, as well as in the mucosal epithelium.

The study confirmed the presence of VPAC1 receptor in the tissues of the porcine female reproductive tract what clearly shows the possibility of influence of VIP on the porcine ovary, oviduct and uterus.

Key words: reproductive system, vasoactive intestinal polypeptide, VPAC1, female, pig

Introduction

Vasoactive intestinal polypeptide (VIP) is regarded to be a co-transmitter in the parasympathetic nerve fibers influencing functions of smooth muscles, glandular tissue and others (Fahrenkrug et al. 1988). It occurs often together with pituitary adenylate cyclase-activating peptide (PACAP) being structurally and functionally similar to VIP. Besides being involved in the neurotransmission VIP (and PACAP) are trophic factors being important in the period of the central nervous system development (Lilling et al. 1994). They are also involved in the neuroprotection (Said et al. 1998, Arciszewski and Ekblad 2005, Arciszewski et al. 2008) and are regarded as potent anti-inflammatory factors (Leceta et al. 2000).

Female reproductive system is supplied by autonomic, afferent and efferent, nerve fibers. They were

Correspondence to: R. Bukowski, e-mail: buki1975@gmail.com

found to contain many neuropeptides, among others neuropeptide Y (NPY), VIP, Substance P (SP), calcitonin gene-related peptide (CGRP), somatostatin (SOM) and enkephalins (Happola et al. 1989, Happola and Lakomy 1989, Happola et al. 1991, Owman et al. 1986). Physiological studies has proven the influence of neuropeptides on the functions of the vagina, uterus and oviduct. VIP increases the bloodflow in the uterus in a dose-dependent manner and decreases its contractility, increases vaginal glands secretion (Ottesen et al. 1983) and increases adenylate cyclase activity in endometrium (Bajo et al. 1993a).

Neurotransmitters require for their action the presence of specific receptor proteins in the target tissues. Initially, the existence of receptors was deduced form pharmacological studies. The application of receptor agonists and antagonists has led to the discovery of many receptors (Langley 1901). The subsequent studies allowed the isolation of receptor proteins, of which the first was nicotinic receptor (Sobel 1977). Neurotransmitter receptors are membrane proteins having the extra- and intracellular part. Neuropeptide receptors belong to the class of "serpentine" receptors coupled to G protein, called also metabotropic receptors. It consists of extracellular part responsible for neurotransmitter binding, seven membrane-spanning domains and the intracellular part responsible for G protein activation. Now, over 150 genes coding for G-coupled receptors are known in the human genome (Hokfelt et al. 2003). Some genes code for receptors whose ligands are still unknown (orphan receptors). Receptors often exist in several sub-types differing in pharmacological properties and distribution.

Receptors for VIP bind also PACAP and some other neuropeptides, like secretin, peptide histidine-isoleucine (PHI) and helodermin (albeit with lower affinity). Now three VIP receptors are known. VPAC1 and VPAC2 bind both VIP and PACAP, while PAC1 receptor binds mainly PACAP (Laburthe et al. 2002). VPAC1 and VPAC2 receptors were found in humans in many tissues, among others in the immune system, lungs, alimentary tract, urogenital and cardiovascular system (Reubi 2000).

The number of receptor molecules may be regulated by alterations of receptor genes expression (up- or down-regulation) (Candenas et al. 2001) or the receptor internalization (Marvizon et al. 1997). Studies of neuropeptide receptors open new possibilities of influencing the organ and tissues functions and, ultimately, developing new effective drugs devoid of common adverse effects. This hope is sparkled by the fact that today ca. 50% of drugs exert their action by interaction with various types of receptors (Drews 2000). For example, NK1 receptor antagonists showed effectiveness in the treatment of depression comparable to Prozac (Holmes et al. 2003).

The studies on the neuropeptide receptors is the field of interest of biochemistry, physiology, pathology and pharmacology. Information on the presence and distribution of receptors in the tissues and organs is very important information both from the point of view of basic sciences and possible applications.

Not much is known of the distribution of neuropeptide receptors in the reproductive organs of the man and laboratory animals. VPAC1 receptor was found in the human reproductive organs (Bajo et al. 2000), but next to nothing is known about the distribution of neuropeptide receptors in the reproductive system of large farm animals. It is why it was decided to study the distribution of VPAC1 receptor in the reproductive organs, the ovary, oviduct and uterus of the pig, the animals regarded often as a species especially suitable as a model animal for studying the human physiology and pathology (Swindle et al. 1992).

Materials and Methods

The study was performed on 8 sexually immature gilts of the Large White Polish breed weighting ca. 25 kg. The study was approved by the Local Ethics Committee. The animals were purchased from the commercial fattening farm. The animals were premedicated with azaperone (Stressnil, Janssen, Belgium; 8 mg/kg of body weight, i.m.) and xylazine (Xylavet, Scanvet, Poland; 2.8 mg/kg of body weight, i.m.) and then the lethal dose of Thiopental (Thiopental, Biochemie, Austria; 40 mg/kg of body weight, i.v.) was administered until the respiratory activity ceased. Animals for morphological studies were then transcardially perfused with 4% solution of paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After perfusion the abdominal cavity was being opened and fragments of the ovary, oviduct, uterine horn and vagina were collected. After 1 h postfixation in the fixative as used for perfusion the tissues were rinsed in phosphate buffer overnight and then transferred to 18% sucrose in phosphate buffer (with 0.1% sodium azide) for storage.

Tissues for morphological studies were cut in a cryostat into 12 μm sections. Sections for immunohistochemistry were put on chrome alum-gelatin-coated slides, air-dried and then stored at -80 Centigrades in air-tight boxes with desiccant.

Animals for molecular biology were dissected without prior perfusion with paraformaldehyde. Fragments of ovaries, oviducts, uterine horns and vagina were excised, weighted, wrapped in Parafilm and alu-

foil and then snap-frozen in liquid nitrogen. Tissues were then stored at -80 Centigrades until further processing.

Total RNA was isolated with TRIzol (GibcoBRL, USA) according to manufacturer instructions. Fragments of tissues weighting 100-200 mg were homogenized with UltraTurrax homogenizer (Janke & Kuhnel, Germany) in 10 ml of TRIzol. After the completed procedure the resulting precipitate of total RNA was dissolved in a nuclease-free water. The concentration of the preparate was determined spectrophotometrically at 260 nm with Ultrospec III UV/VIS spectrophotometer (LKB, Sweden). The purity of the preparate was determined by a A_{260}/A_{280} ratio which had to be in the range 1.6-2.0. The preparates of total RNA were stored at -80 Centigrades. The cDNA synthesis was performed as described elsewhere (Wasowicz 2003).

Since the sequence of porcine NK1 mRNA is unknown starters for PCR were designed on the basis of the analysis of homology of NK1 mRNA sequences of other species available in GenBank. The homology analysis was performed with ClustalW software. Sequences used for homology analysis were: human NK1 receptor (M74290), murine NK1 receptor (X62934), guinea pig SP receptor (X64323) and rat SP receptor (M31477). Transcript fragments with homologous sequences were identified and the starter sequences were picked up. The sequence of sense starter was CAGCCACGGCTACCAA, and the sequence of antisense starter was GCTTGAAGCCCAGACG. The melting temperature of both starters was 54 Centigrades. The expected length of PCR product was 544 base pairs (bp).

PCR reaction was performed as described elsewhere (Wasowicz 2003). 30 cycles were performed with a thermal profile of 95 Centigrades denaturation for 10 s, 54 Centigrades starter hybridization for 10 s and 72 Centigrades DNA synthesis for 30 s. PCR products were analysed in 0.8% agarose gel in TAE buffer. The size of PCR product was assessed with a DNA size marker (M1, DNA Gdansk, Poland). DNA fragments were visualized with ethidium bromide (EtBr, 10 mcg/ml of gel) and an UV transluminator with a wavelength of 302 nm. Gels were documented with a video camera, equipped with appropriate filters, coupled to PC computer equipped with an image grabber and image acquisition software.

Immunoblotting

Frozen fragments of the ovary, oviduct, uterine horn and cervix were homogenized in impact homogenizer (Kucharczyk TE, Poland). Pulverized tissues were transferred to the lysis buffer consisting of 2% SDS, 50 mM TRIS (pH 6.8), 100 mM di-thiotreitol (DTT, Sigma, USA), 10% glycerol (POCh, Poland) and 0.1% bromophenol blue (POCh, Poland). The volume of the buffer was adjusted to 100 mg tissue per 1 ml of the buffer. Tissues were homogenized with an UltraTurrax homogenizer (Janke & Kuhnel, Germany). Then the homogenate was heated to 95 Centigrades for 5 min, cooled to room temperature and centrifuged for 2 min at 12000g. The proteins were separated, blotted and detected as described elsewhere (Wasowicz 2003).

Immunohistochemistry

Immunohistochemical stainings were performed on slides taken out from the freezer and then air-dried at RT for 20 min. Every section was outlined with a DAKOPEN marker (DAKO, Denmark). Then slides were processed as described elsewhere (Wasowicz 2003). The primary antibody was monoclonal mouse anti-VPAC1 antibody (Cat. No. V1631; Sigma, USA) (1:200). The secondary antibody was biotinylated goat anti-mouse antibody (Cat. No. E043, DAKO, Denmark) (1:400).The secondary antibody was detected with Cy3-conjugated streptavidin (Cat. No. 016-160-084, Jackson Immunoresearch Labs, USA) (1:4000). Then slides were examined with a Axiophot fluorescent microscope (Zeiss, Germany) with a filter set specific for Cy3. Sections were imaged using a laser scanning confocal microscope (MRA-2, Biorad, UK).

Staining specificity was verified by control stainings in which the primary antibody was either omitted, or replaced with a normal non-immune rabbit serum. In none of the control stainings any signal was detected.

Results

The application of starters specific for VPAC1 receptor in RT-PCR performed on RNA isolated from the ovary, oviduct, uterine horn and cervix resulted in all the organs with the presence of the specific band of the expected size – ca. 486 bp (Fig. 1). The intensity of the bands in all organs was comparable.

Application of VPAC1 receptor antibody for immunoblot on extracts from the ovary, oviduct, uterine horn and cervix showed the presence of a protein band of the size of ca. 52 kDa alongside with several weak bands (Fig. 2).

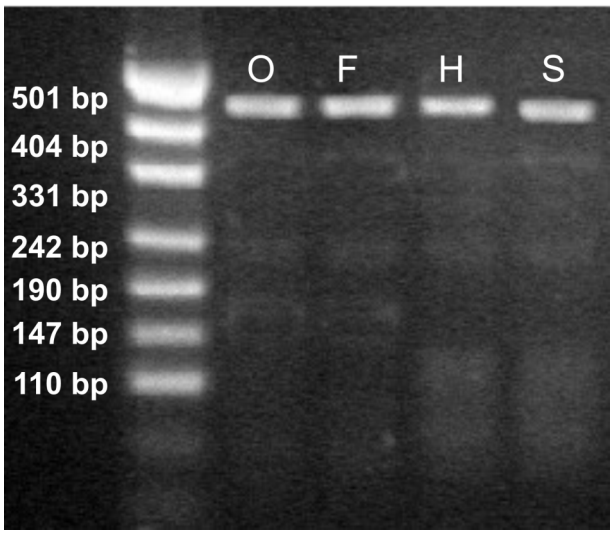

Fig. 1. Agarose electrophoresis of VPAC1 receptor PCR product (486 bp). O – ovary, F – oviduct, H – uterine horn, S – uterine cervix

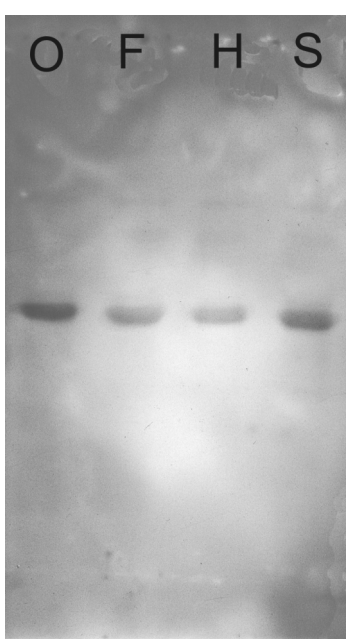

Fig. 2. Immunoblot of protein extracts from the porcine reproductive system. The antibody detected a protein with a molecular mass of 52 kDa. O – ovary, F – oviduct, H – uterine horn, S – uterine cervix

In the ovary immunochemistry detected the presence of VPAC1 receptor in the muscular membrane of blood vessels, mainly arteries, rarely in veins. Some arterial blood vessels were devoid of the staining for VPAC1 receptor (Fig. 3a). Weak staining was visible in the wall of primary ovarian follicles (Fig. 3b), while stronger staining was seen in the granulosa of later stages of ovarian follicles, as well as in oocytes (Fig. 3cd). In the oviduct the staining for VPAC1 receptor was visible in the arterial blood vessels located in the muscular membrane and submucosa (Fig. 4a). The staining was also visible in the smooth muscle cells of the muscular membrane of the oviduct (Fig. 4b). The staining was also visible in the apical parts of the mucosal epithelium (Fig. 4c).

In the uterine horn the staining for VPAC1 receptor in the blood vessels was very weak (Fig. 5a). Similarly, the staining in the muscular membrane of the uterine horn was weak and was visible as patches of immunoreactivity (Fig. 5b). In the uterine glands the staining for VPAC1 receptor was seen in the apical (luminal) parts of the epithelial cells (Fig. 5c). VPAC1 staining was also visible in the epithelial cells of the mucosa.

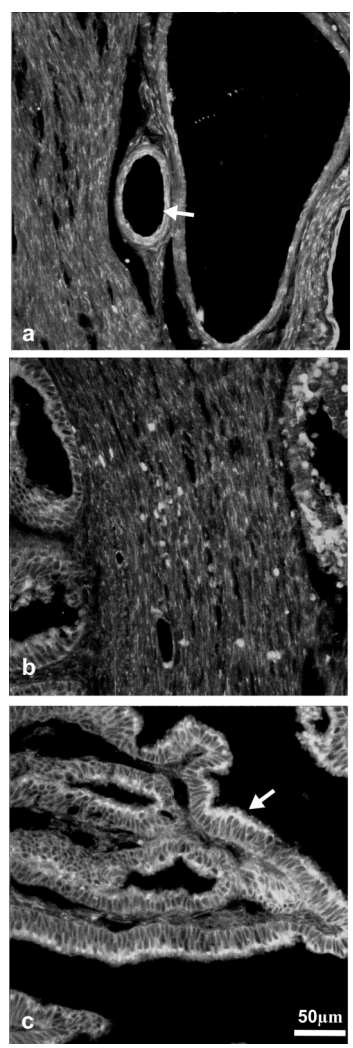

Fig. 4. A. Oviduct isthmus. Immunostaining for VPAC1 receptor in the blood vessels; B. Oviduct isthmus. Immunostaining for VPAC1 receptor in the muscular membrane; C. Oviduct isthmus. Immunostaining for VPAC1 receptor in the mucosal epithelium. Mag. 150x

In the uterine cervix VPAC1 staining was visible in the muscular membrane of larger arteries. In the muscular layer VPAC1 receptor staining was visible

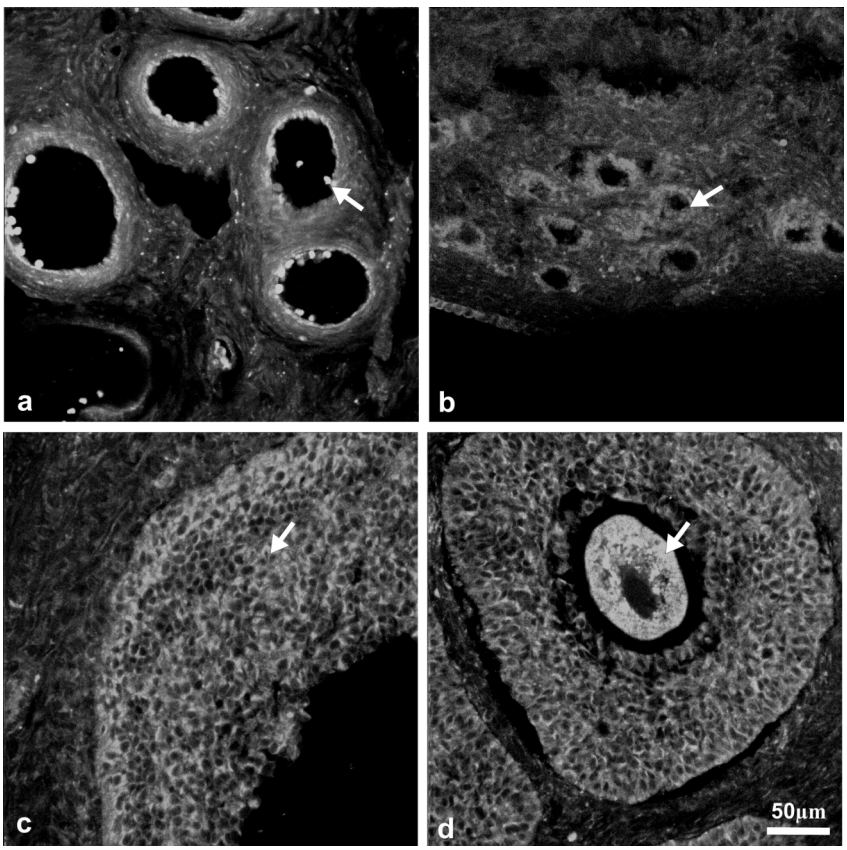

Fig. 3. A. Ovary. Immunostaining for VPAC1 receptor in the blood vessels (arrow): B. Ovary. Immunostaining for VPAC1 receptor in the primary ovarian follicles (arrow); C. Ovary. Immunostaining for VPAC1 receptor in the granulosa cells (arrow); D. Ovary. Immunostaining for VPAC1 receptor in the oocyte (arrow). Mag. 150x

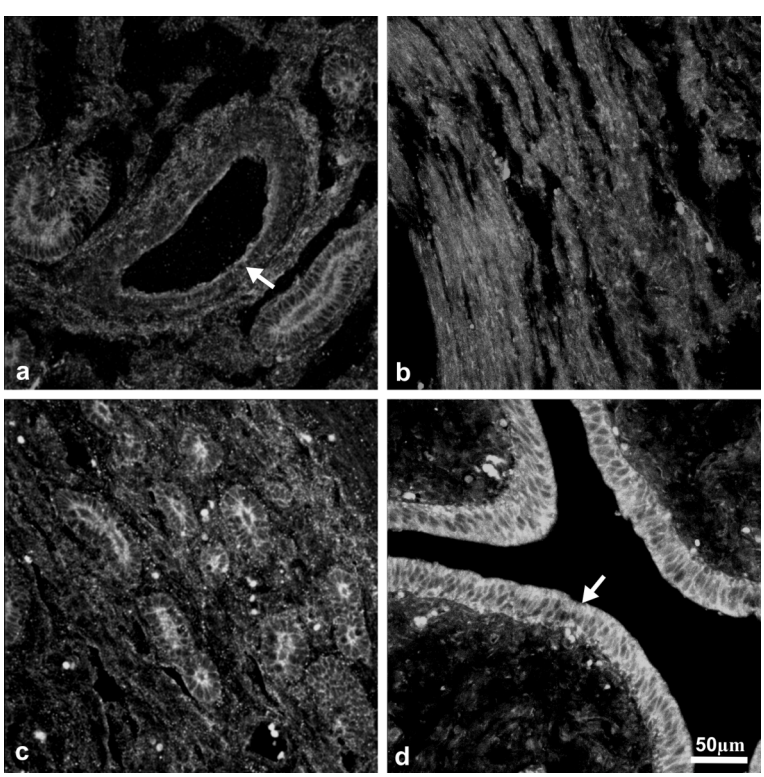

Fig. 5. A. Uterine horn. Immunostaining for VPAC1 receptor in the blood vessels (arrow); B. Uterine horn. Immunostaining for VPAC1 receptor in the muscular membrane (arrow); C. Uterine horn. Immunostaining for VPAC1 receptor in the submucosa (arrow); D. Uterine horn. Immunostaining for VPAC1 receptor in the mucosal epithelium (arrow). Mag. 150x

only in some areas, giving it an appearance of patches. VPAC1 receptor staining was also visible in the cells of mucosal epithelium (data not shown).

Discussion

VIP was found to have a variety of functions. It influences blood flow (Altiere and Diamond 1983), functions of the immune system (Ganea 1996), the course of inflammatory processes (Abad et al. 2003), proliferation of cancer cells (Chen et al. 1994) and many others. The presence of nerve fibers containing VIP in the female reproductive organs is known (Owman and Stjernquist 2003), however, there was no interest in the distribution of VIP receptors in these organs. More attention was paid to the receptors for sex steroids and gonadotropins (Ikeda and Inoue 2004). There is lack of studies which systematically investigate the distribution of VIP receptors in the female reproductive organs.

RT-PCR delivered the expected 486 bp PCR product confirming the expression of VPAC1 receptor in the ovary, oviduct, uterine horn and cervix. Although the intensity of the PCR product bands in all the studied parts of the porcine reproductive system was identical no quantitative conclusions about the VPAC1 receptor expression can be drawn. VPAC1 receptor was detected with RT-PCR in the central nervous system and peripheral tissues in humans (Gkonos et al. 2000) and rat (Vertongen et al. 1997). This method was seldom used to study the VPAC1 receptor expression in the reproductive system (Garcia-Fernandez et al. 2003) and the papers dealing with this problem in female genital organs are absent.

The method complementary to RT-PCR enabling detection of receptor proteins at the tissue level is immunoblot. However, the antibodies available are usually directed to human or rat proteins and there is risk that the proteins of other species may be non-detectable due to the aminoacid sequence differences. However, application of the commercial anti-VPAC1 receptor antibody in the immunoblot analysis of proteins extracted from the four studied organs of the porcine female genital tract detected the protein band with the apparent molecular mass of 52 kDa, while the calculated molecular mass of the mouse VPAC1 receptor is 52,1 kDa. Immunoblot was used to detect the VPAC1 receptor in the tissues of the laboratory animals and humans (Garcia-Fernandez et al. 2003,Pozo et al. 2000) but there are no such studies in other animal species. Immunoblot, in addition to the specific band of the expected molecular mass, visualized additional, weaker, bands which may correspond to alternative molecular forms of the receptor proteins, truncated or alternatively glicosylated (Assil and Abou-Samra 2001). In the mouse VPAC1 receptor protein 5 potential sites of glycosylation were identified.

Morphological studies on the presence and distribution of neuropeptide receptors in the peripheral tissues are scarce (Goto et al. 1998).

The role of neuropeptide receptors on the functions of tissues may be deduced from the presence of nerve fibers containing the particular neuropeptide and from the pharmacological effect of the neuropeptide in that tissue. However, this conclusions cannot regard the molecular characterization of the receptor subtypes.

VIP-positive nerve fibers in the ovary of sexually immature gilts were comparatively scarce and located under the capsule, around ovarian follicles and blood vessels (Majewski et al. 1995). In the oviduct and uterus of the sexually mature sows the VIP-positive nerve fibers were located in the muscular membrane and in the mucosa as free-ranging or blood vessels associated ones (Wasowicz et al. 1998,Wasowicz et al. 1999).

In the present study VPAC1 receptor was detected in all the studied parts of the porcine female reproductive system. In the ovary it was found mainly in the walls of the blood vessels, however, weak staining was visible also in the walls of the ovarian follicles at different stages of maturation. In the literature only scarce papers dealing with the VIP/PACAP receptors can be found. In the human ovary the VIP binding and VPAC1 expression (with immunoblot) were shown (Bajo et al. 2000) as well as the binding of VIP by the hen granulosa cells (Kawashima et al. 1995). It is known that in the ovary VIP stimulates the synthesis of cAMP, estradiol and progesterone (Davoren and Hsueh 1985). In the mouse VIP stimulates also the growth and maturation of ovarian follicles (Cecconi et al. 2004).

VPAC1 receptor was shown in the muscular membranes of the oviduct and two studied parts of the uterus, in the blood vessel walls, glandular cells of the uterine horn and in the cells of the mucosal epithelium. There are no papers dealing with the morphological distribution of VPAC1 receptor in the oviduct and uterus. In the human oviduct and uterus only the VIP binding and VPAC1 receptor protein band of 47kDa expression by immunoblot were detected (Bajo et al. 2000). However, physiological studies clearly show that, for example, VIP increases in a dose-dependent manner the blood flow through the rabbit uterine muscular membrane (Ottesen 1981). The way of VIP action on the blood vessels is only partially elucidated. Some reports suggest the direct influence of the peptide on the smooth myocythes (Barnes et al.

1986), while other reports suggest indirect influence, via the endothelium-produced NO (Jovanovic et al. 1998).

VIP stimulates cAMP synthesis in the human cultured endometrial cells what suggests its influence on the functions of the endometrium (Bajo et al. 1993b). The presence of VPAC1 receptor in the cells of the endometrial epithelium may be surprising, however VIP binding as found in the mucosal epithelium of the human gastrointestinal tract (Zimmerman et al. 1989).

The distribution of VIP receptors was not studied in the tissues of the oviduct and uterus but it is known that this peptide inhibits the activity of the muscular layer cells of the oviduct (Bredkjoer et al. 1997) and uterus (Ottesen 1981). The binding of VIP was found to be hormone-dependent, as estradiol with progesterone were augmenting VIP binding in the ovariectomized rabbits (Ottesen et al. 1985).

VIP is also an important trophic factor for many cell populations (Waschek 1995) and VIP receptors are present in many tumors of "reproductive" origin (Garcia-Fernandez et al. 2003) and in the breast cancer cells (Waschek et al. 1995).

The results presented here are the first comprehensive ones dealing with the expression and distribution of VPAC1 receptor in the tissues of the reproductive system of large farm animals. They may be used as a basis and supplementation for pharmacological and physiological studies on the influence of VIP on the female genital organs and the influence of various factors on the expression of VPAC1 receptor in the ovary, oviduct and uterus.

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