Immunohistochemical characterisation of cholinergic nerve fibres supplying accessory genital glands in the pig

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ABSTRACT: Our previous immunohistochemical investigations revealed three major populations of nerve fibres supplying the porcine accessory genital glands (AGG) including noradrenergic, non-noradrenergic putative cholinergic and sensory nerve terminals (Kaleczyc et al., 1997). However, it is still unclear whether the non-noradrenergic nerve fibres are cholinergic in nature. The knowledge of the population of cholinergic nerve fibres in mammalian AGG based upon vesicular acetylcholine transporter (VAChT) immunohistochemistry is very limited. Therefore, the aim of the present investigation was to disclose the occurrence and colocalization patterns of VAChT, dopamineβ-hydroxylase (DβH) and some neuropeptides including vasoactive intestinal polypeptide (VIP), neuropeptide Y (NPY) and somatostatin (SOM) within nerve fibres supplying the porcine AGG. Double-immunohistochemical labellings showed that VAChT-positive nerve terminals were non-adrenergic (DβH-negative), however, many of them contained immunoreactivities to VIP, NPY and/or SOM. The coexistence patterns of these biologically active substances in nerve fibres supplying particular glands are similar but the density of cholinergic innervation varies between the organs. The innervation of the seminal vesicle and prostatic body is better developed than that of the disseminated part of the prostate and bulbourethral glands. The majority of cholinergic nerve fibres associated with blood vessels supplying the glands contain VIP and NPY and, to a lesser degree, SOM. The possible function and origin of the cholinergic nerve fibre population are discussed.

Keywords: accessory genital glands; cholinergic innervation; neuropeptides; immunohistochemistry; pig

In the boar, accessory genital glands include the body and disseminated part of the prostate, paired seminal vesicles and bulbourethral glands. These glands receive an abundant innervation arising primarily from pelvic ganglia and, to a lesser extent, from the inferior mesenteric ganglion as well as from the sympathetic chain and dorsal root ganglia (DRG) (Sjostrand, 1965; Costa and Furness, 1973; Kaleczyc et al., 1993, 1994, 2002; Kolbeck and Steers, 1993; Dhami and Mitchell, 1994; Danuser et al., 1997).

Two groups of autonomic nerves are thought to control the secretory function of AGG (Dail, 1993). The expulsion of glandular excretions during seminal emission is thought to be largely under sympathetic-noradrenergic control. Noradrenergic nerves induce contractions of smooth muscle cells surrounding the secretory epithelium. Sympathetic non-adrenergic (putative cholinergic) nerves are believed to be secretomotor to the glandular epithelium.

It has been demonstrated in a variety of species including the rat, mouse, hamster, guinea-pig, cat, rabbit, dog and humans that nerves innervating AGG, in addition to classical neurotransmitters such as acetylcholine and noradrenalin, may also contain other biologically active substances including substance P (SP), metenkephalin (Met-ENK),

leu-enkephalin (leu-ENK), neuropeptide Y (NPY), vasoactive intestinal peptide (VIP), somatostatin (SOM), calcitonin gene-related peptide (CGRP) and nitric oxide (Alm et al., 1980, 1981; Stjernquist et al., 1987; Aumuller et al., 1989; Higgins and Gossling, 1989; Crowe et al., 1991; Kepper and Keast, 1995; Jen et al., 1995; Tainio, 1995; Hedlund et al., 1996; Chow et al., 1997; Sjostrand et al., 1998).

The knowledge of neurochemical properties of adrenergic nerve fibres supplying mammalian AGG is comprehensive and detailed because methods used to investigate adrenergic nerve structures are well developed and considered to be reliable. On the other hand, until quite lately, the exact identification of peripheral cholinergic nerve structures was impossible because specific markers to investigate this part of the peripheral nervous system (PNS) were not available. The most commonly used histochemical method for localization of acetylcholinesterase (AChE) is thought to be controversial in assessment of cholinergic nerve fibres because this enzyme has been found to be related not exclusively to cholinergic axons but also to nerve terminals belonging to other subdivisions of the PNS (Koelle, 1955; Burn and Rand, 1965; Jacobovitz and Koelle, 1965). Recently, efficient antibodies against an isoform of choline acetyltransferase (ChAT; an enzyme of acetylcholine synthesis) found in the peripheral nerve structures were raised and characterised, and using these antisera the presence of ChAT-containing nerve fibres in the wall of the vas deferens was revealed (Kujat et al., 1993; Kaleczyc et al., 2000). Moreover, an alternative marker for cholinergic nerve structures has become available in the form of an antibody to vesicular acetylcholine transporter (VAChT – the protein associated with the storage of acetylcholine within vesicles in cholinergic nerve terminals). VAChT-positive nerve fibres were found in the human prostate and seminal vesicle (Dixon et al., 2000) and in the rat prostate (Schafer et al., 1998; Nadelhaft, 2003). However, the knowledge of neurochemical properties of cholinergic nerve fibres supplying AGG is still very limited. Therefore, the present study was designed to investigate the presence and coexistence of VAChT and other biologically active substances including NPY, VIP, SOM (neuropeptides found earlier to occur most frequently in nonadrenergic nerve fibres supplying the porcine AGG; Kaleczyc et al., 1999) and DβH in nerve terminals supplying the porcine AGG using single and double-labelling immunofluorescence.

MATERIAL AND METHODS

The experiment was performed on three juvenile (12–15 kg body weight, b.w.) and three adult (90–100 kg b.w.) boars of the Large White Polish race obtained from a commercial fattening farm in Purda (Poland). All the animals were housed and treated in accordance with the rules approved by the local Ethics Commission (conforming the principles of Laboratory Animal Care, NIH publication No. 86-23, revised in 1985).

Removal, fixation and sectioning of AGG. Prior to perfusion, the pigs were deeply anaesthetised. Thirty minutes before the main anaesthetic pentobarbital (Vetbutal, Biowet, Poland; mg/kg b.w.) was applied intravenously, all the animals were pretreated with propionylopromazine (Combelen; Biowet, Poland 0.7 mg/kg b.w., *i.m.*). Then they were transcardially perfused with 0.5 l (juvenile animals) or 1 l (adult boars) of preperfusion solution containing 0.9% sodium chloride (Chemia, Gliwice, Poland), 2.5% polyvinylpyrrolidone (Sigma, Deisenhofen, Germany), 0.5 procaine hydrochloride (Polfa, Warsaw, Poland) and 20 000 IU of heparin (Heparinum; Polfa; added ex tempore), followed by 4 l (juvenile animals) or 8 l (adult boars) of 4% ice-cold buffered paraformaldehyde (pH 7.4).

Samples of bulbourethral glands, seminal vesicles and prostates (including the body and disseminated part) were dissected out and postfixed by immersion in the same fixative for 2 hours, then rinsed with phosphate buffer (pH 7.4) and transferred to and stored in18% buffered sucrose solution (pH 7.4) until further processing. Eight-µm cryostat sections from the glands were cut longitudinally or transversally. All the sections were mounted on chrome alum-gelatine-coated glass slides.

The sections were processed for single- or double-labelling immunohistochemistry to study the presence and coexistence of VAChT with other biologically active substances including DβH, VIP, NPY and SOM in nerve fibres supplying the glands.

Immunohistochemical procedure. The sections were air-died at room temperature (RT) for 30 min, rinsed (3 × 5 min) with phosphate-buffered saline (PBS; pH 7.4), preincubated with a blocking mixture containing 0.25% Triton X-100, 1% bovine serum albumin and 10% normal goat serum in PBS (1 h, room temperature, RT), and incubated overnight (ON) in a humid chamber with one (single labellings) or a mixture of two (double-labellings) primary antisera raised in different

species. Afterwards, in the case of single-labellings, the sections were incubated with an appropriate biotinylated antiserum (1h, RT, for single- and double-labellings this step was sometimes omitted) followed by incubation with CY-3-conjugated streptavidin or an appropriate fluorescein isothiocyanate (FITC)-conjugated secondary antiserum (1h, RT); in the case of double-stainings, mixtures of the appropriate secondary reagents mentioned above were used. Primary antisera and secondary reagents are listed in Table 1. Each step of immunolabelling was followed by raising the sections with PBS (3 × 5 min). Finally, they were mounted with carbonate-buffered glycerol (pH 8.6). The stained sections were studied with Zeiss-Axiophot microscope equipped for epifluorescence and an appropriate filter set for CY3 and FITC.

Controls. Preabsorption of the diluted antiserum with 20 ug/ml of the appropriate antigen (besides DβH) completely abolished the specific immunoreaction. Additionally, the primary antisera were omitted or replaced by non-immune sera or by PBS in order to check the method specificity.

RESULTS

Because no distinct differences were found in the innervation of AGG between the juvenile and adult pigs, the results are described together. Moreover, double-labelling investigations revealed no colocalization of VAChT and DβH in nerve fibres supplying the glands, thus the nerve terminals immunoreactive to these substances formed two separate populations of axons.

Distribution of VAChT-immunoreactive (VAChT-IR) nerve fibres supplying the seminal vesicles and their chemical coding

Single-labelling immunofluorescence disclosed that the seminal vesicle was intensely supplied with VAChT-positive, varicose nerve fibres. The varicose nerve terminals were often observed to occur beneath but never penetrating into the epithelium. They were also related to smooth muscle cell bundles located in the interstitial tissue of the glands (Figures 1, 2, 3a).

Both intrinsic as well as extrinsic arteries were moderately supplied with VAChT-IR nerve fibres while veins were apposed by solitary nerve terminals only.

Double-labelling immunofluorescence revealed that many VAChT-IR nerve fibres supplying the seminal vesicle contained also NPY, and some of them stained also for VIP or SOM. Most of VAChT-positive nerve terminals located beneath the tubular epithelium displayed immunoreactivities to NPY, VIP or SOM (Figures 1, 2, 3). The vast majority of VAChT-IR fibres found in the interstitial tissue was simultaneously NPY- or VIP-positive

Table 1. List of primary antisera and secondary reagents used in the study

Antigen	Code	Dilution	Species	Supplier
Primary antibodies				
VAChT	H-V006	1 : 8 000	rabbit	Phoenix Pharmaceuticals, USA
NPY	NZ1115	1 : 150	rat	Affiniti, UK
SOM	8330-0009	1 : 50	rat	Biogenesis, UK
VIP	MaVIP	1 : 1 500	mouse	East Acres, USA
Secondary reagents				
Biotinylated anti-rabbit IgG		1 : 400	goat	Dako, Denmark
Biotinylated anti-rat IgG		1 : 400	rabbit	Dako, Denmark
Biotinylated anti-mouse IgG		1 : 400	goat	Dako, Denmark
FITC-conjug. anti-mouse IgG		1 : 400	goat	Jackson Immun. Lab. USA
FITC-conjug. anti-rat IgG		1 : 50	goat	Jackson Immun. Lab. USA
Streptavidin-conjug. C_Y3		1 : 4 000		Dianova, Hamburg, Germany

Table 2. Coexistence patterns of VAChT and neuropeptides in nerve fibres supplying the porcine accessory genital glands and semiquantitative evaluation of their relative frequency

Coexistence pattern	Seminal vesicle		Prostatic body		Disseminated prostate		Bulbourethral gland		A	V
	BE	IT	BE	IT	BE	IT	BE	IT		
VAChT/NPY	++	+++	+++	+	++	+/-	++	+/-	+	+/-
VAChT/SOM	+	+/-	+/-	+	+	+/-	+	+/-	+/-	+/-
VAChT/VIP	+	++	++	+	++	+/-	++	+	++	+/-

BE – beneath the epithelium, IT – interstitial tissue, A – arteries, V – veins, – not found, +/– single fibres, + few fibres, ++ moderate number of fibres, +++ many fibres

but only the solitary nerve endings exhibited immunoreactivity to SOM (Figure 3). Most of the perivascular VAChT-IR nerve fibres stained also for NPY or VIP.

Distribution of VAChT-IR nerve fibres supplying the prostate and their chemical coding

Single-labelling immunofluorescence revealed that immunoreactivity to VAChT was expressed in numerous nerve fibres supplying the prostatic body while they were less frequently encountered in the disseminated part of the gland. VAChT-IR nerve terminals closely apposing glandular tubules were more numerous than those associated with the smooth muscle cell bundles in the interstitial tissue (Figures 4, 5, 6). Both intrinsic and extrinsic arteries were moderately supplied with VAChT-positive nerve fibres. Veins were only occasionally apposed by the solitary axons.

Double-immunostaining revealed the presence of immunoreactivity to NPY, VIP and SOM within VAChT-IR nerve fibres supplying the body and disseminated part of the prostate. Many VAChT-positive nerve terminals, especially those located beneath the tubular epithelium, contained NPY- and VIP-immunoreactivity (Figures 4, 5), however, only solitary nerve terminals stained also for SOM (Figure 6). The majority of VAChT-IR perivascular nerve fibres also displayed immunoreactivity to

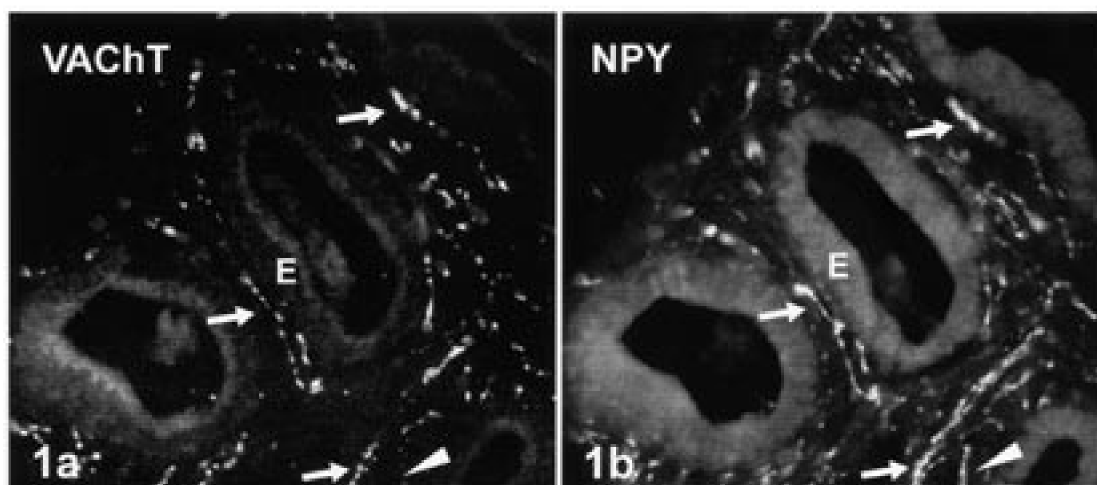

Figure 1. The seminal vesicle in the juvenile boar. Vesicular acetylcholine transporter (VAChT)- [**a** CY3 visualisation (CY3)] and neuropeptide Y-immunoreactive (NPY-IR) [**b** fluorescein isothiocyanate visualisation (FITC)], nerve fibres (*arrows*) associated with the glandular epithelium (E) or located within the interstitial tissue (IT) contained both substances studied. In contrast, NPY-positive but VAChT-negative nerve fibre (arrowhead) was located within the interstitial tissue (IT). Magnification 450×

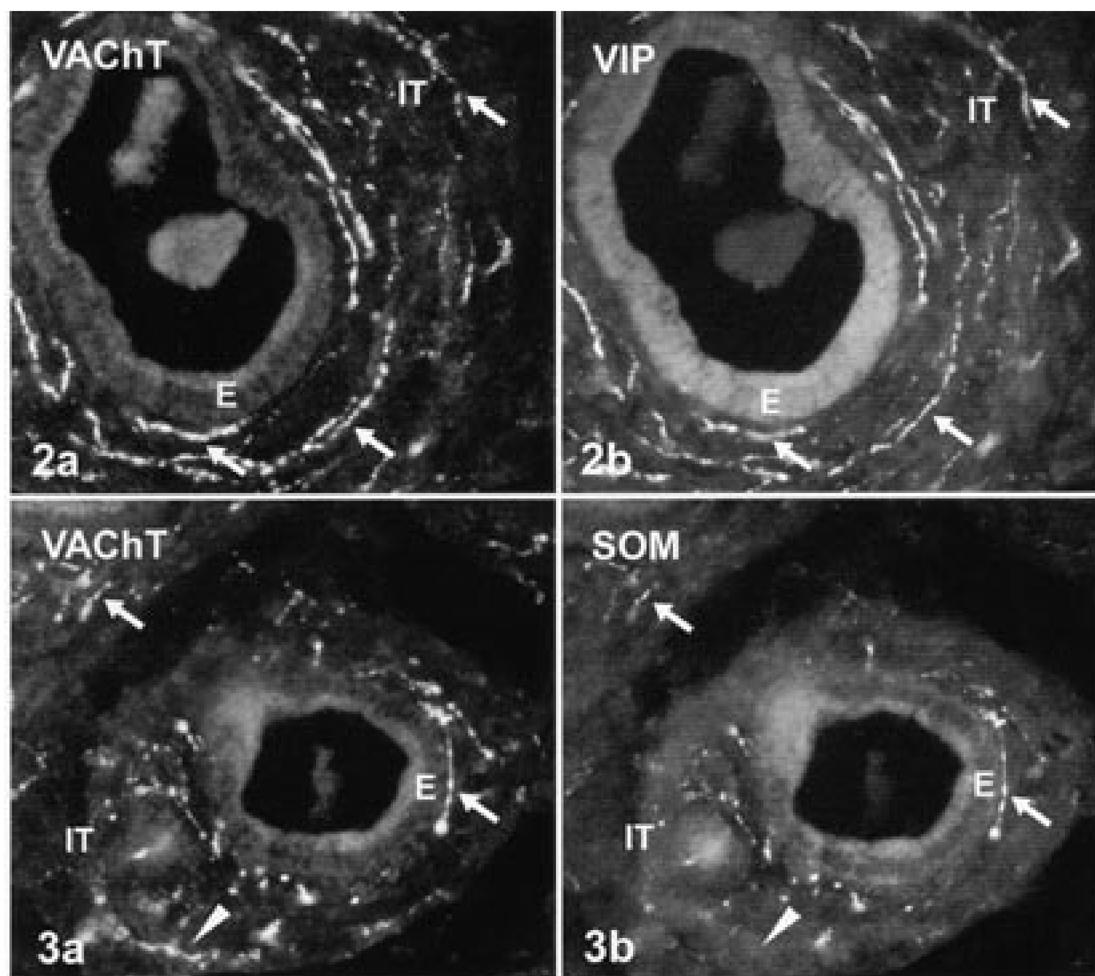

Figure 2. The seminal vesicle in the juvenile boar. VAChT- (**a** CY3) and vasoactive intestinal polypeptide (VIP)-IR (**b** FITC) nerve fibres (*arrows*) associated with the glandular epithelium (E) or located within the interstitial tissue (IT). Magnification 450×

Figure 3. The seminal vesicle in the juvenile boar. VAChT-IR (**a** CY3) and somatostatin (SOM)-positive (**b** FITC) nerve fibres (arrows) associated with the glandular epithelium (E) or interstitial tissue (IT). In contrast, VAChT-positive (**a**) but SOM-negative (**b**) nerve fibre (*arrowhead*) was located within the interstitial tissue (IT). Magnification 450×

NPY and VIP, and the solitary nerve endings were simultaneously SOM-positive.

Distribution of VAChT-IR nerve fibres supplying the bulbourethral gland and their chemical coding

As revealed by single-labelling immunofluorescence, VAChT-positive nerve fibres moderately supplied the bulbourethral gland. These nerve terminals were often found beneath the tubular epithelium while they were less numerous within the interstitial tissue (Figures 7, 8, 9). Both intrinsic and extrinsic arteries were moderately supplied with VAChT-positive nerve fibres. Veins were only occasionally apposed by the solitary axons.

Double-labelling investigations revealed that many VAChT-IR nerve terminals supplying the bulbourethral gland, especially those located beneath the epithelium, contained also NPY-, VIP- or SOM-immunoreactivity (Figures 7, 8, 9). Most of the perivascular VAChT-containing nerve fibres also displayed immunoreactivity to NPY or VIP and the solitary axons were simultaneously SOM-positive.

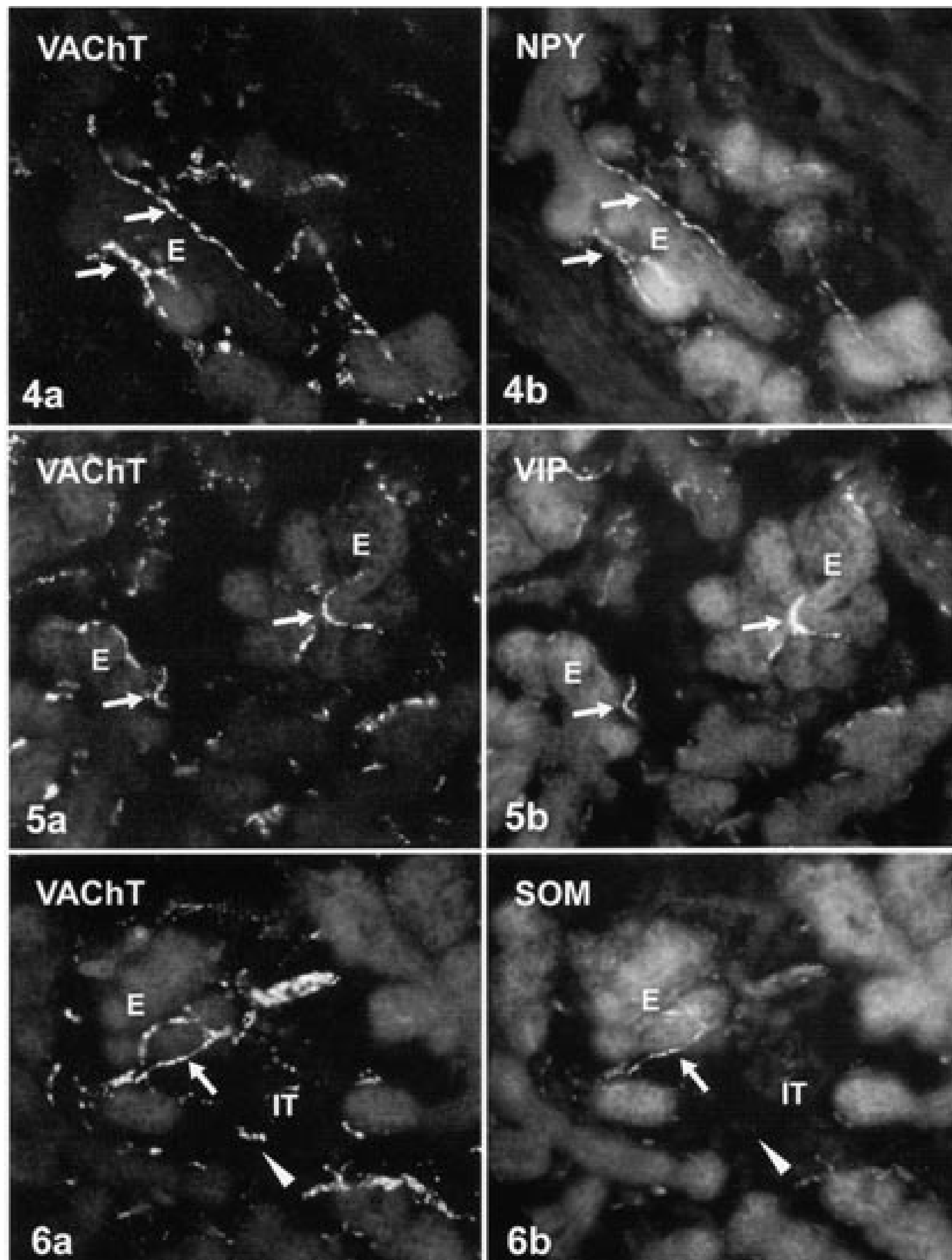

Figure 4. The disseminated part of the prostate in the juvenile boar. VAChT-IR (**a** CY3) nerve fibres (*arrows*) associated with the glandular epithelium (E) also contained neuropeptide Y (NPY)-IR [**b** FITC]. Magnification 450×

Figure 5. The disseminated part of the prostate in the juvenile boar. The majority of VAChT-IR (**a** CY3) nerve terminals (arrows) supplying glandular acini stained also for VIP (**b** FITC). Magnification 450×

Figure 6. The disseminated part of the prostate of the juvenile boar. VAChT-IR (**a** CY3) and somatostatin (SOM)-positive (**b** FITC) nerve fibres (arrow) associated with the glandular epithelium (E). In contrast, VAChT-positive (**a**) but SOM-negative (**b**) nerve fibre (*arrowhead*) was located within the interstitial tissue (IT). Magnification 450×

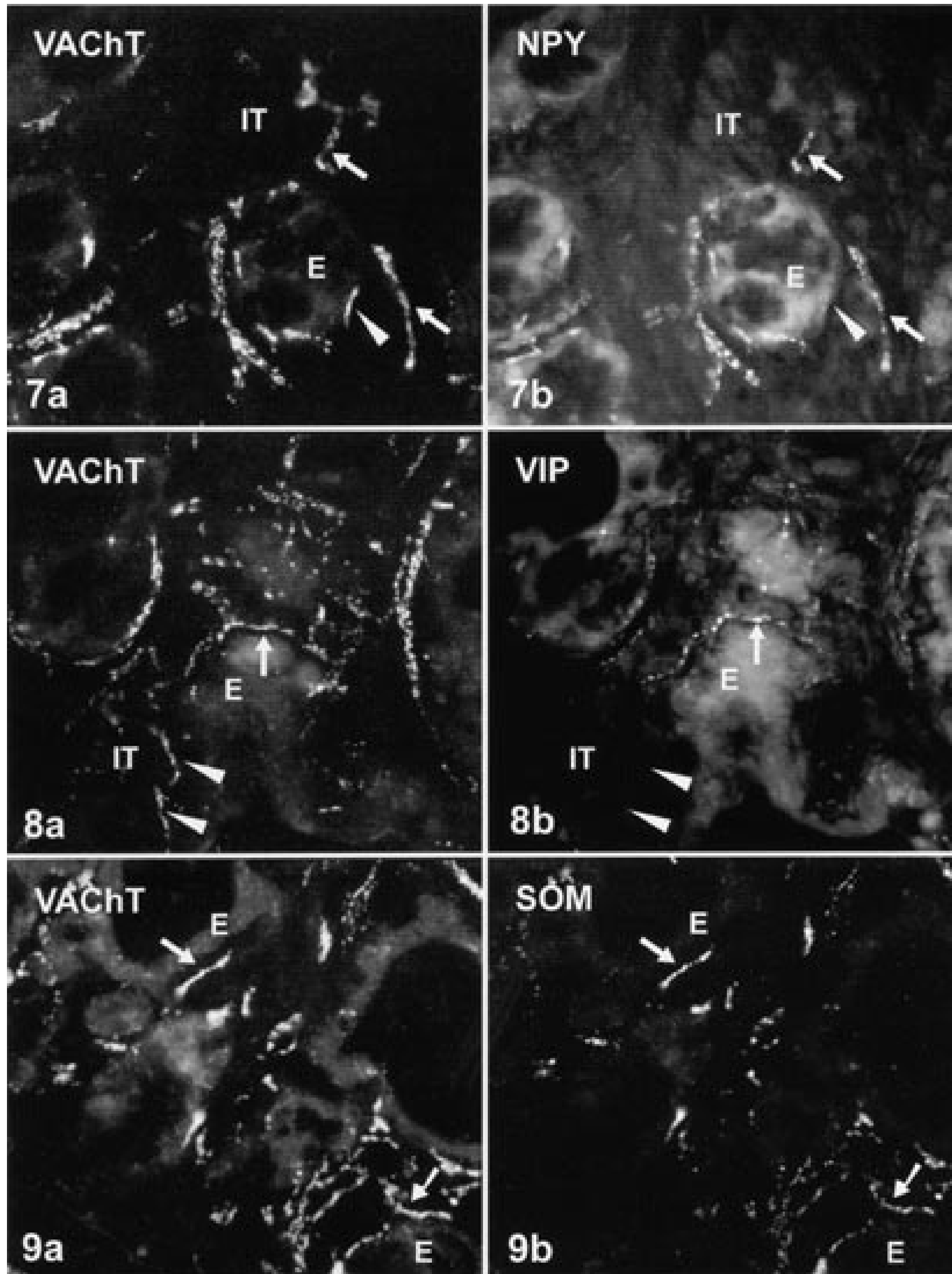

Figure 7. The bulbourethral gland in the juvenile boar. VAChT- (**a** CY3) and NPY-IR (**b** FITC) nerve fibres (*arrows*) running in the interstitial tissue between the tubules of the gland. In contrast, VAChT-positive (**a**) but NPY-negative (**b**) varicose nerve fibre (*arrowhead*) was located beneath the epithelium of the tubule. Magnification 450×

Figure 8. The bulbourethral gland in the juvenile boar. VAChT- (**a** CY3) and VIP-IR (**b** FITC) varicose nerve fibre (*arrow*) associated with the glandular epithelium. Some VAChT-positive nerve fibres (arrowheads) located within the interstitial tissue (IT) were VIP-negative. Magnification 450×

Figure 9. The bulbourethral gland in the juvenile boar. VAChT- (**a** CY3) and SOM-IR (**b** FITC) nerve fibres (*arrows*) associated with the glandular epithelium (E). Magnification 450×

DISCUSSION

The present study has shown that the porcine AGG are supplied by moderate numbers of nerve fibres containing immunoreactivity to VAChT, therefore they should be regarded as cholinergic nerve terminals. The VAChT- positive nerve fibres closely apposing the tubules of the glands are more numerous than those associated with smooth muscle cell bundles in the interstitial tissue. The general distribution of cholinergic nerve terminals in the porcine AGG is similar to that of AChE-positive axons found in other mammals including the hamster, guinea pig, rat and humans (Al-Zuhair et al., 1975; Dunzerdorfen et al., 1976; Vaalasti and Hervonen, 1979; Moss et al., 1987; Higgins and Gosling, 1989; Vega Alvarez et al., 1989; Chow at al., 1997). The literature dealing with the population of cholinergic nerve fibres supplying mammalian AGG based upon VAChT immunohistochemistry is very limited. The presence of cholinergic (VAChT-positive) fibres was shown in the human prostate and seminal vesicle (Dixon et al., 2000) and in the rat prostate (Schafer et al., 1998; Nadelhaft, 2003). Dixon et al. (2000) found that the majority of VAChT-positive nerve endings localized beneath the epithelium of the human prostate and seminal vesicle contained also NPY whereas the small proportion stained for nitric oxide synthase (NOS).

In our recent study dealing with the innervation of the porcine AGG we found that many non-adrenergic nerve fibres were localized beneath the tubular epithelium and in the interstitial tissue (Kaleczyc et al., 1999). These fibres coexpressed immunoreactivities to NPY and VIP, VIP and SOM or to NPY and SOM, thus it is likely that these tree peptides coexist within some of the nerve terminals. The present investigations using single- and double-immunofluorescence confirmed these findings and revealed that these non-adrenergic nerve terminals were cholinergic in nature (VAChT-positive).

The biologically active substances found to coexist within cholinergic nerve fibres supplying porcine AGG probably perform an inhibitory function with regard to their smooth muscle cells and blood vessels. VIP has been demonstrated in nerve fibres supplying the prostate and seminal vesicles in the guinea-pig, rat, cat, rabbit, mouse and humans. VIP-immunoreactive nerve fibres have been mainly associated with the basis of the glandular epithelium and, in smaller proportion, with the smooth muscle coat and blood vessels (Alm et al., 1980; Vaalasti et al., 1980, 1986; Larsen et al., 1981; Gu et al., 1983; Stjernquist et al., 1983; Polak and Bloom, 1984; Lamano Carvalho et al., 1986; Higgins and Gosling, 1989; Lange and Unger, 1990; Properzi et al., 1992; Jen et al. 1995; Kepper and Keast, 1995; Tainio, 1995). Very little is known about the physiological role of VIP in AGG. Pinho et al. (1994) reported that in the seminal vesicle of the hamster VIP-IR nerve fibres also manifested AChE activity and suggested a modulatory function of this peptide on muscarine receptors. VIP is typically present in cholinergic nerves in other tissues (Ottesen and Fahrenkrug, 1995). On the other hand, there is a widespread agreement that cholinergic nerves are secretomotor to the glandular epithelium of the prostate and seminal vesicles (Dail, 1993). Moreover, normal rat prostate epithelial cells have been shown to contain VIP receptors and to produce cAMP in response to the action of this peptide (Carmena and Prieto, 1985a,b). It is therefore possible, that VIP plays a role in the regulation of the secretory function of epithelial cells in this and other AGG. Immunoreactivity to SOM was found in a few nerve fibres supplying the human seminal vesicle and prostate (Gu et al., 1983; Chapple et al., 1991; Crowe et al., 1991; Tainio, 1995). Mirabella et al. (2003) revealed SOM-IR nerve fibres innervating the prostate in the water buffalo. In this species non-vascular SOM-positive axons contain neither VIP nor TH, thus these fibres probably belong to a subpopulation of non-adrenergic nerves distinct from those containing NOS, VIP and NPY.

The presence of NPY-positive nerve endings in the reproductive organs which receive a dense sympathetic nerve supply such as the vas deferens and accessory sex glands was reported earlier. In the seminal vesicle of man (Adrian et al., 1984; Lange and Unger, 1990; Tainio, 1995) and rodents (Dhami and Mitchell, 1984; Lamano Carvalho et al., 1986; Stjernquist et al., 1987; Yuri, 1990; Properzi et al., 1992; Irvani and Zar, 1994) NPY-immunoreactive nerve fibres appear to be the major peptide-containing neuronal component. These fibres have been predominantly found in the smooth muscle layer of the glands, however, some of them have been detected in the subepithelial tissue and in association with blood vessels. The rich supply of NPY-IR nerve fibres in the non-vascular smooth muscle of accessory sex glands certainly suggests a role in the muscular control of secretory output. Stjernquist et al. (1987) found that NPY largely collocalized with DβH within axons in the guinea-pig seminal

vesicles. This observation was confirmed by Dhami and Mitchell (1994), who found that in this species many pelvic adrenergic neurons projecting to the seminal vesicles, but not to the prostate, contained this peptide. Kepper and Keast (1995), in turn, revealed that many, if not all adrenergic nerve fibres innervating the rat prostate contained NPY.

It is commonly accepted that in mammals, pelvic ganglia are the main source of nerve fibres supplying male reproductive organs (Sjostrand, 1965; Keast, 1992; Kolbeck and Steers, 1993). Kaleczyc et al. (2003) revealed that nearly all non-adrenergic neurons in the porcine APG (Anterior Pelvic Ganglion) were cholinergic in nature, i.e. expressed immunoreactivity for ChAT and VAChT, substances commonly accepted as specific markers of cholinergic nerve structures. These nerve cells co-express simultaneously immunoreactivities to other biologically active substances including NOS, VIP, NPY and SOM. Therefore it is very likely that cholinergic nerve fibres observed in the present study are processes of these neurons. Nadelhaft (2003) also demonstrated small cholinergic (VAChT-positive) neurons in the rat pelvic ganglion supplying the prostate gland.

The literature in the field contains some data dealing with the chemical coding of non-adrenergic pelvic neurons in mammals. It seems to be noteworthy that there are profound species-dependent variations with regard to the expression of biologically active substances in pelvic non-adrenergic nerve cells. Keast (1991) revealed that in the male rat two populations of cholinergic neurons in the major pelvic ganglion (MPG) could be distinguished. The first consisted of cholinergic NPY-positive neurons (stain only for NPY) and the second one was represented by cholinergic VIP-IR neurons (stain only for VIP). No SOM-IR MPG neurons have been found in this species. Thus, in contrast to the pig, NPY- and VIP-positive nerve cell bodies form separate populations of rat male pelvic cholinergic somata that do not contain SOM. In human male infants and children, non-adrenergic pelvic neurons display VIP-, NPY-, NOS-, SOM-, CGRP- and SP-immunoreactivity (Jen et al., 1996). Because the vast majority of non-adrenergic nerve cells in human pelvic ganglia contains VAChT (Dixon et al., 1999), these neurons should be considered as cholinergic.

This is the first comprehensive study dealing with the chemical coding of cholinergic nerve fibres supplying the porcine AGG. These nerve terminals are mostly associated with the tubular epithelium and contain a variety of collocalized biologically active substances including VAChT as well as NPY, VIP or SOM. Considering a great coincidence between the chemical coding of cholinergic nerve fibres supplying the porcine AGG and that of cholinergic pelvic neurons it is further concluded that pelvic ganglia are the major source of cholinergic innervation for the porcine urogenital system.

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