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Immunolocalization of regulatory peptides and 5-HT in bovine male urogenital apparatus

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Summary. Specimens of testis, excurrent duct including the accessory genital glands and urethra throughout its extension were investigated in adult bovines, in order to immunohistochemically localize both the peptidergic innervation and the epithelial cell types belonging to the diffuse endocrine system (DES). Immunoreactivities to GRP, met- and leu-enkephalins, CGRP, NPY, substance P, VIP, somatostatin, ß-endorphin and 5-HT antisera were tested by means of a labelled streptavidin-biotin (LSAB) method. Such regulatory substances were found in components of the peripheral nervous system (nerve fibers in the connective and muscular tissues, sub- and intraepithelial nerve terminals, nerve cell bodies and fibers in intramural ganglia), and in epithelial endocrine/paracrine cells. Bovine urogenital apparatus is supplied by many peptide-containing nerves, which contain in many localizations GRP and enkephalins, and to a lesser extent substance P, CGRP, NPY and VIP. A thin network of peptidergic nerves distributes to the musculature of the canalicular organs and accessory glands. The prostatic complex was especially rich in peptidergic innervation, and also contained somatostatin- and 5-HT-secreting endocrine cells. In addition, 5-HT-immunoreactive endocrine cells were found in the bulbourethral gland and urethral epithelium. CGRP-ir nerves were present contacting striated muscle fibers of urethra (motor end plates). The testis was devoid of any immunoreactivity. These data are compared with those obtained in a companion study carried out the same organs in two species of *Equidae* (*Equus caballus* and *Equus asinus*). Different patterns of immunoreactivities can be outlined in these domestic ungulates.

Key words: Regulatory peptides, 5-HT, Endocrine cells, Male urogenital apparatus, *Bos taurus*

Introduction

It is now well-known that chemically different neuromediators exist both in the central and peripheral nervous systems in addition to the neurotransmitters. These accessory neuromediators were first described in the central nervous system and gastrointestinal tract (brain-gut axis), and thereafter also found in other apparatuses in the nerves supplying the various organs (Burnstock et al., 1979; Polak and Bloom, 1980). They are reputed neuromodulators, in that they may show excitatory or inhibitory actions towards the responsiveness of the target structures to the neurotransmitters. These neuromodulators often coexist with the classical neurotransmitters in the same nerve fiber (Burnstock, 1976; Burnstock et al., 1979; Lundberg and Hökfelt, 1983). In addition, some of these regulatory substances may be localized to the recepto-secretory cells belonging to the Diffuse Endocrine System. These cells are characterized by basally-located membrane-bound granules containing peptides, amines or other messengers and acidic carrier proteins including chomogranins. Because of this secretory portion they exert an endocrine (blood-borne) or paracrine (within a short distance) activity. They also possess a receptive portion, by which they can adequately recognize stimuli at the cell surface (Fujita, 1989).

In recent years, the study of the peptidergic innervation and the description of endocrine/paracrine cells in the male and female urogenital apparatuses has received much attention (Gu et al., 1983; Huang et al., 1984; Lamano Carvalho et al., 1986; Fahrenkrug et al., 1989; Lakomy et al., 1995; Majewski et al., 1995; Houdeau et al., 1997). In male genital organs, several neuropeptides were immunohistochemically demonstrated in nerves supplying both the canalicular organs and the glandular complexes (for a review, see Arrighi and Domeneghini, 1997). The presence of endocrine-like cells in the male urogenital tract has been described in the human since 1951 (Feyrter, 1951). In this species, the prostate is especially rich in endocrine/paracrine cells, possibly related to pathological conditions (Di Sant'Agnese and De Mesy Jensen, 1984; Di Sant'Agnese, 1986; Di Sant'Agnese and Cockett, 1994; Speights et al., 1994). In addition, endocrine-like cells have been described in laboratory species and in some domestic mammals too (Hanyu et al., 1987; Iwanaga et al., 1987; Cecio and Vittoria, 1989; Vittoria et al., 1990;

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Arrighi and Domeneghini, 1997).

Because of our interest in the comparative study of the distribution of regulatory substances in the urogenital apparatus, in the present paper we refer about the presence of nine peptides and the biogenic amine 5-hydroxytryptamine throughout the male genital apparatus of *Bos taurus*, with the aim of comparing the pattern identified in the bovine male genitalia with that of other ungulates, such as *Equidae* (Arrighi and Domeneghini, 1997).

Materials and methods

Several specimens of testis, epididymis, *ductus deferens* and *ampulla ductus deferentis*, vesicular gland, prostate, bulbourethral gland, as well as pelvic (prostatic and non-prostatic) and penile urethra were collected at slaughtering from 4 healthy male bovines, 20-24 months old.

The fixation and sectioning of the specimens, as well as the immunohistochemical procedures on sections were performed as previously described in detail (Arrighi and Domeneghini, 1997). Briefly, the samples were fixed by immersion overnight at 4 °C in 4% paraformaldehyde in phosphate-buffered saline (PBS) 0.1M at pH 7.4, then snap-frozen in iso-Pentane cooled by liquid nitrogen. 12 to 14 μm-thick cryostat sections were immunostained both after an enzyme (bovine trypsin) treatment aimed to unmask the antigens and increase the immunostaining of the substances tested, or without this step. Sections were immunostained by incubating them with the primary antisera (first layer) listed in Table 1.

Each primary serum was used at various dilutions until the optimal one was found (see results). Incubations were carried out in a moist chamber at 4 °C for 18-22 h. For the immunohistochemical procedure a labelled streptavidin biotin (LSAB) system was employed, with biotinylated swine anti-rabbit immunoglobulins (Dako, Italy) as secondary serum, followed by a Strept-ABComplex/HRP (Dako). Tris-buffered saline (TBS: 0.05M Tris/HCl, 0.15M NaCl) pH 7.6, was used for dilutions and rinses throughout the whole procedure. 3-amino-9-ethylcarbazole (AEC) was employed as chromogen. Sections were counterstained with Mayers' hematoxylin and mounted using Glycergel (Dako), an aqueous mounting medium.

The positive control was performed using sections of gut which were collected from the same bovines.

The negative controls were performed by (1) the use of non-immune rabbit serum in place of specific antisera; (2) incubating sections with antisera pre-absorbed with the respective antigens (Sigma, Italy); and (3) omission of the first layer. All of them resulted in the absence of immunoreaction.

Slides were observed and photographed under an Ortoplan photomicroscope (Leitz, Germany). Estimates of staining intensities and quantitative evaluations of immunoreactive structures were performed by both the authors, after examination of many sections per organ or region of the male urogenital apparatus in all the animals tested.

Results

The regulatory peptides were extensively present in nerve components in different localizations of the bovine urogenital apparatus. Immunoreactive nerve fibers running in the connective tissue or in relation to muscle cells, sub- and intraepithelial nerve terminals, nerve cell bodies and fibers in intramural ganglia were frequently shown. The somatostatin peptide and 5-HT were exclusively found in epithelial endocrine cells.

Table 2 summarizes the results obtained in the different organs.

Since the amino acid sequence of bovine peptides is not known and a cross-reactivity with unknown peptides containing the same antigenic sequences cannot be excluded, the immunoreactive material is referred to as "peptide-like" immunoreactivity (ir).

GRP/Bombesin-like-immunoreactivity

GRP/Bombesin-like-ir nerve structures (Fig. 1) were extensively found in the *ductus deferens* and its *ampulla*. They were present in a smaller quantity in the *vesicular*

Table 1. Primary antisera tested, all raised in rabbits.

PRIMARY ANTISERA TESTED	SOURCE	CODE
Anti-synthetic gastrin releasing peptide (GRP)/Bombesin*	Amersham, UK	RPN 1692
Anti-synthetic methionine-enkephalin	Amersham, UK	RPN 1562
Anti-leucine-enkephalin	Peninsula, UK	RAS 8601 N
Anti-human calcitonin gene-related peptide (CGRP)	Peninsula, UK	RAS 6009 N
Anti-rat calcitonin gene-related peptide (CGRP)	Peninsula, UK	RIN 6006
Anti-synthetic neuropeptide Y (NPY)	Amersham, UK	RPN 1702
Anti-synthetic substance P	Amersham, UK	RPN 1572
Anti-porcine vasoactive intestinal peptide (VIP)	Amersham, UK	RPN 1582
Anti-synthetic somatostatin	Amersham, UK	RPN 1612
Anti rat ß-endorphin	Peninsula, UK	RAS 8843 N
Anti-rat 5-hydroxytryptamine (5-HT)	Peninsula, UK	61066

*: this anti-GRP serum does not cross-react with Substance P (Manufacturer's declaration).

gland and *prostate* (body), and were sporadically seen in the *bulbourethral gland* and *epididymis*. Thin immunoreactive nerve fibers were observed in the epithelial layer of *urethra*. In the *ductus deferens* and its *ampulla* GRP-like-ir nerves were present as thin single fibers distributed throughout the muscle layer, where they ran in close vicinity to the muscle cells (Fig. 1a). Immunoreactive nerve fibers were additionally present just beneath the ampullary glandular and surface epithelia (Fig. 1b). Intensely immunoreactive nerve fiber bundles were seen at the periphery of these organs, below the serous membrane (Fig. 1c). In the *corpus prostatae* and *vesicular gland* (Fig. 1d) nerve fiber bundles surrounded the glandular lobules and insinuated

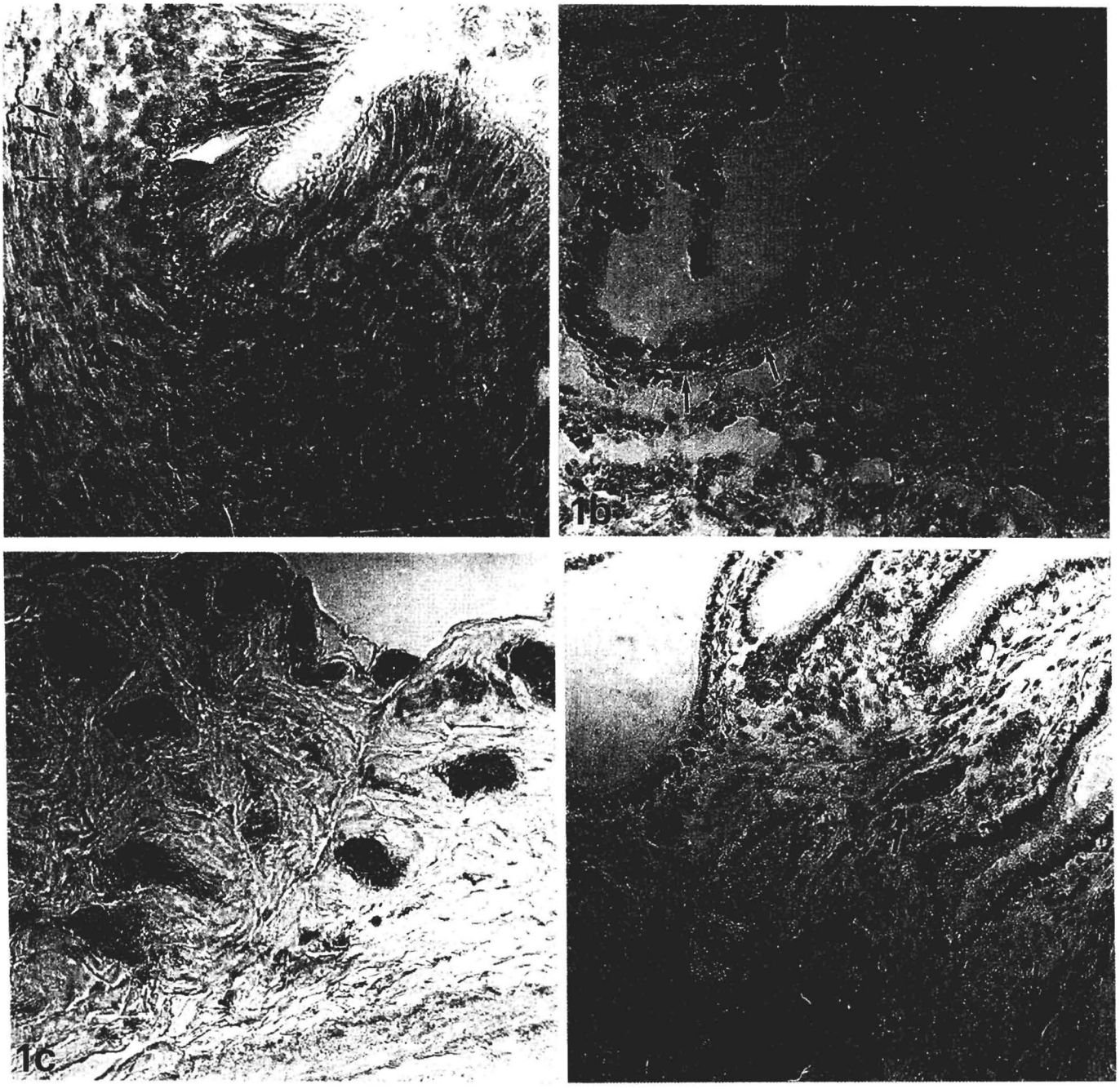


Fig. 1. GRP/Bombesin-like immunoreactivity in bovine genitalia. **a.** In the ductus deferens GRP-ir nerves (arrows) are thin and run in close relationship to the muscle cells. x 330. **b.** GRP-Ir nerves are localized just beneath the glandular epithelium of ampulla (arrows). x 330. **c.** GRP-ir nerve fiber bundles are present in subserosal location of ductus deferens. x 200. **d.** In the vesicular gland GRP-ir nerve fiber bundles are present in the interstitial spaces (thick arrows). In some places they run in close vicinity to the glandular epithelium (thin arrows). x 200

into the interstitial spaces reaching the glandular epithelium, being often in contact with the muscular component. Limited to the prostatic body, GRP-like-ir was found in nerve cell bodies and fibers of intramural ganglia.

Enkephalin-like-immunoreactivity

[met]- and [leu]-enkephalin-like-ir (Fig. 2) showed a similar pattern of distribution in the bovine urogenital apparatus. The difference was that leu-enkephalin-like-ir was less abundant if compared with the companion peptide in nerve structures and was not identifiable in epithelial cells of the DES, which, on the contrary, showed met-enkephalin-like ir in the *prostate*. The two antisera marked numerous nerve fibers in the muscular coat of *ductus deferens* and its ampulla (Fig. 2a), where, in addition, sub-epithelial nerve fibers were shown (Fig. 2b). In the *vesicular gland*, sparse enkephalin-like-ir nerve fibers were localized especially in the connective tissue between the adenomers. Numerous immunoreactive nerve fibers were found in the prostatic complex, in close vicinity to the muscular and epithelial components (Fig. 2c). In addition, prostatic intramural ganglia were shown which contained numerous immunoreactive nerve bodies and fibers, as well as immunoreactive nerve terminals which encircled immunonegative neurons (Fig. 2d).

CGRP-like-immunoreactivity

CGRP-like-ir was present in thin solitary nerve fibers, sparsely localized within the musculature or epithelium, both in the prostatic complex (Fig. 3a) and in the *ductus deferens*, being less numerous in its ampulla. Prostatic intramural ganglia showed CGRP-like-ir nerve fibers which apposed immunonegative neurons. Throughout the extension of the *urethra*, sub- and intraepithelial immunoreactive terminals were detectable (Fig. 3b). In the *tunica muscularis* of the pelvic urethra, thin arborizing nerve terminals were seen (Fig. 3c) contacting striated muscle fibers. They strictly resembled motor end plates (MEPs).

NPY-like-immunoreactivity

NPY-like-ir was intense in nerves of the smooth muscular coat of *ductus deferens*. The immunoreactive terminals also insinuated within the mucosal *plicae*. In other localizations, such as vesicular and bulbourethral glands, as well as urethra (Fig. 3d), reactivity was present in extremely thin nerve fibers which ran solitary or in small bundles (Fig. 3d, insert) near the epithelium. In the *corpus prostatae*, NPY-like-ir was localized in intramural ganglia, both in neurons and nerve terminals.

Substance P-like-immunoreactivity

Substance P-like-ir was present in extremely thin nerve fibers in the *ductus deferens* (Fig. 4a). Sparse subepithelial immunoreactive nerve fibers were seen in the *prostate* and *bulbourethral gland*. Limited to prostatic complex, immunoreactive nerve fibers were seen in perivascular localizations regarding small arteries and arterioles.

VIP-like-immunoreactivity

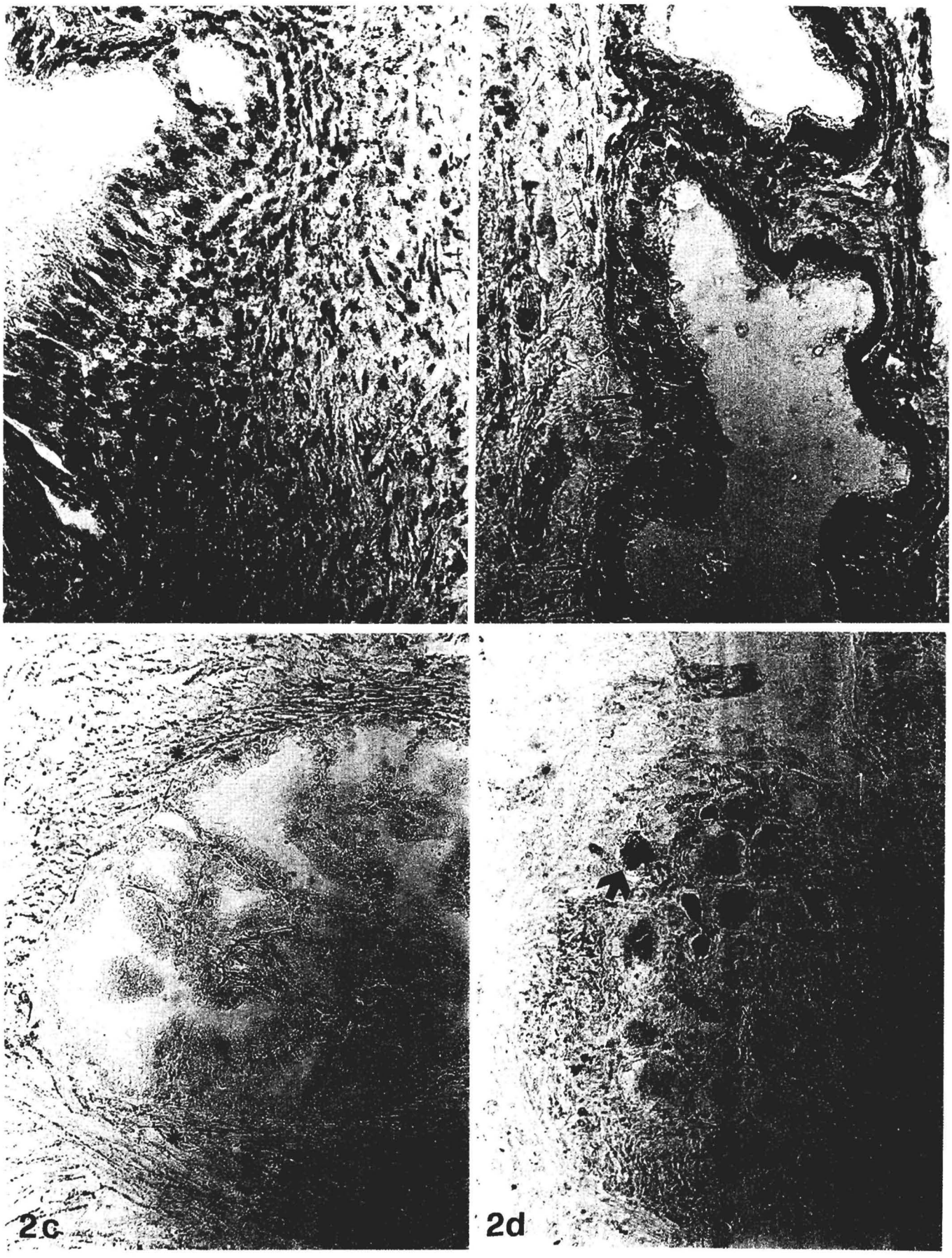
VIP-like-ir was almost exclusively localized in the

Table 2. Immunolocalization of regulatory peptides and 5-HT in the male urogenital organs of *Bos taurus*. Optimal dilutions of antisera are indicated.

	GRP/BOMB 1:600	[met]-ENK 1:800	[leu]-ENK 1:2000	CGRP 1:5000	NPY 1:1200	SUB P 1:600	VIP 1:1000	SOM 1:1000	ß-END 1:3000	5-HT 1:3000
Testis	-	-	-	-	-	-	-	-	-	-
Epididymis	$\pm^{a}$	$+^{a}$	$+^{a}$	-	-	-	-	-	-	-
Ductus deferens	$+++^{a}$	$+++^{a}$	$++^{a}$	$+^{a}$	$++^{a}$	$+^{a}$	-	-	-	-
Ampulla	$+++^{a}$	$+++^{a}$	$++^{a}$	$\pm^{a}$	-	-	-	-	-	-
Vesicular gland	$++^{a}$	$+^{a}$	$+^{a}$	-	$\pm^{a}$	-	-	-	-	-
Corpus prostatae	$++^{a,b}$	$\pm^{DES}/+++^{a,b}$	$++^{a,b}$	$+^{a,b}$	$\pm^{a,b}$	$\pm^{a,c}$	$+^{a}$	$\pm^{DES}$	-	$++^{DES}$
Pars disseminata	-	$\pm^{DES}/++^{a,b}$	$+^{a,b}$	$++^{a,b}$	$\pm^{a}$	$\pm^{a,c}$	$+^{a}$	$\pm^{DES}$	-	$+^{DES}$
Bulbourethral gland	$\pm^{a}$	$\pm^{a}$	$\pm^{a}$	-	$\pm^{a}$	$\pm^{a}$	$\pm^{a}$	-	-	$+^{DES}$
Urethra	$+^{e}$	-	-	$++^{d,e}$	$\pm^{a}$	-	-	-	-	$+++^{DES}$

Quantification of immunoreactive structures is indicated as: ±, weak or unevenly present reaction; +, reaction detectable in a small number of structures; ++, reaction detectable in a medium number of structures; +++: strong reaction detectable in a large number of structures; unreactivity is indicated as -. [DES]: epithelial cells of the diffuse endocrine system; [a]: nerve fibers in connective or smooth muscle tissues; [b]: neurons and /or nerve fibers in intramural ganglia; [c]: perivascular nerve fiber; [d]: motor end plates in striated tunica muscularis; [e]: intraepithelial nerve fibers.

Fig. 2. [met]-Enkephalin-like-immunoreactivity in bovine genitalia. **a.** In the ductus deferens numerous enk-ir nerve fibers can be seen in the muscular coat (arrows). x 290. **b.** In the ampulla deferentis strongly enk-ir nerve fibers are present in sub-epithelial localizations (arrows). x 360. **c.** In the corpus prostatae thin enk-ir nerve fibers are present either in close relationship to the muscle cells (asterisks) or in subepithelial localizations (arrows). x 200. **d.** In a prostatic intramural ganglion numerous enk-ir nerve bodies (thick arrows) and fibers (thin arrows) are present. x 310


2c
2d

prostatic complex where immunoreactive nerve fibers were present in large bundles, from which thin nerves distributed through the interstitial spaces to the glandular epithelia (Fig. 4b). *The bulbourethral gland* received this innervation too, but to a lesser extent.

Somatostatin-like-immunoreactivity

Somatostatin-like-ir was seen in the *prostatic complex*, where it was localized exclusively to a very small number of endocrine cells. These were distributed

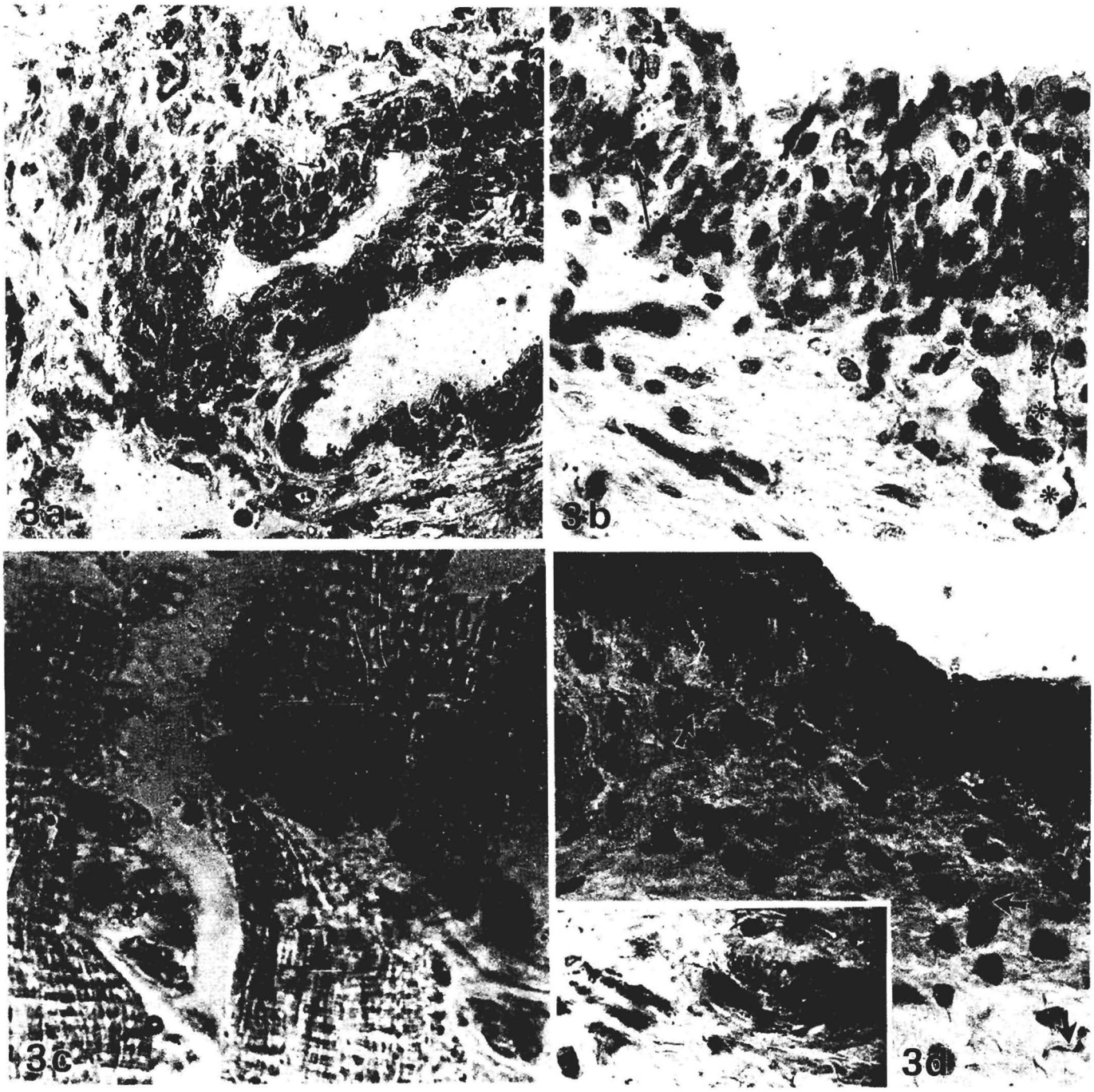


Fig. 3. a. CGRP-like ir nerve fibers (arrows) can be seen within the glandular epithelium of the disseminate part of the prostate. x 400. **b.** CGRP-ir nerve terminals (arrows) are present within the epithelium of the pelvic urethra or just beneath it (asterisks). x 540. **c.** A CGRP-ir arborizing nerve terminal, likely to be a motor end-plate (curved arrow), is present in the striated tunica muscularis of pelvic urethra. x 1,440. **d.** Tiny solitary NPY-like ir nerve fibers (arrows) can be seen just beneath the epithelium of prostatic urethra. x 580. Insert: In the same localization nerve fibers are seen running in small bundles. x 770

among the glandular epithelial cells and were round or ovoidal.

ß-Endorphin-like-immunoreactivity

ß-endorphin-like-ir was never detected throughout the bovine male urogenital appartus.

5-HT-immunoreactivity

This was exclusively detected in epithelial endocrine cells (Fig. 5). These were seen in the *prostatic complex*, being more numerous in the corpus, and the *bulbo-urethral gland* (Fig. 5a), where they distributed among the cells of the glandular epithelium. In addition, a large number of these endocrine cells was seen in the epithelium lining the urethra (Fig. 5b), throughout its extension. Above all when seen in the urethral localizations, they were slender and extremely polymorphic owing to their cytoplasmic projections insinuating among other epithelial cells. This morphological aspect suggests a possible paracrine functional role. The majority of these endocrine cells reached the lumen, thus suggesting a functional role towards the reception of chemical stimuli in relation to the passage of urina and/or sperma.

Discussion

Within the different organs of the bovine urogenital apparatus, only the testis never showed any immunoreactivity. Within the different regulatory substances tested, ß-endorphin alone was never immunohistochemically identified. Somatostatin neuropeptide and 5-HT biogenic amine have been exclusively found in epithelial components of DES. Somatostatin is present in the prostatic complex, 5-HT in the prostatic complex, in the bulbourethral gland and, with a greater abundance, in the urethral epithelial layer.

A network of peptidergic nerves distributes to the musculature of the canalicular organs, especially ductus deferens and its ampulla. The musculature of the accessory genital glands is also richly innervated by peptidergic nerve fibers, which often insinuate into interstitial spaces reaching the glandular epithelia, or their vicinity. Among the accessory glands, the prostatic complex is particularly supplied by nerve terminals releasing many neuropeptides.

The peptidergic nerve terminals have been shown to release primarily GRP and enkephalins, and, to a lesser extent, CGRP, NPY, substance P and VIP. With the exception of the nerves which release substance P and VIP, the peptidergic nerve terminals may in some instances originate from intrinsic neurons of intramural ganglia, which in some localizations are immunoreactive to GRP, enkephalins, CGRP and NPY.

The presence of GRP-ir nerve fibers is described in ductus deferens and vesicular gland of mouse, rabbit and guinea-pig (Stjernquist et al., 1983), whereas Gu et al. (1983) failed to find this peptide throughout the human

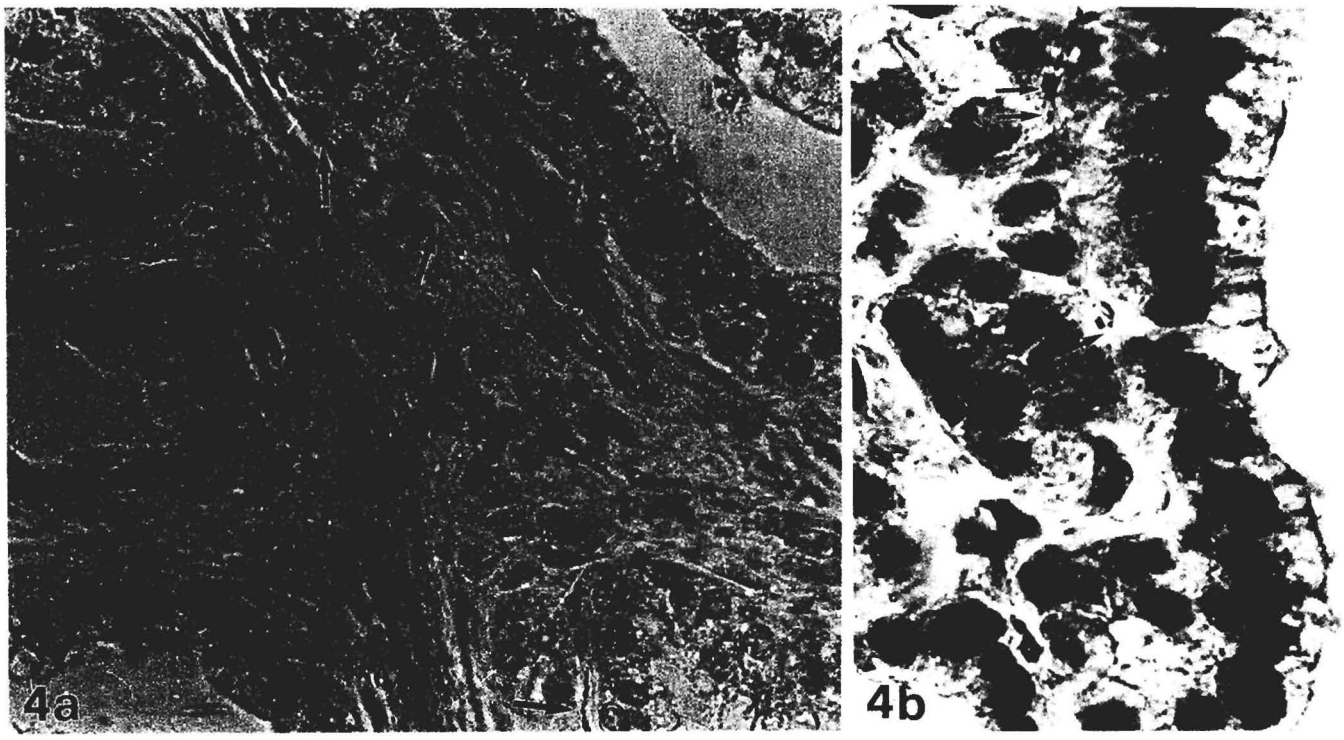


Fig. 4. a. Substance P-like-immunoreactivity can be seen in thin nerve fibers in the corpus prostatae (arrows). x 640. **b.** VIP-like-ir nerve fibers are evident in subepithelial localization in prostatic urethra (arrows). x 1,150

male genitalia. In *Equidae*, we detected some GRP-immunoreactive nerve fibers in the muscular coat of the ductus deferens and, more rarely, in perivascular localizations (Arrighi and Domeneghini, 1997). Surprisingly, the female genital tract of the same species is devoid of bombesin/GRP-ir nerve terminals (Lakomy et al., 1995). This different pattern in the male and female genital tract of the same species has an unknown significance, at present. This discrepancy might be explained away by the different methods employed to reveal antigen-antibody complexes (streptavidin-biotin immunohistochemistry versus immunofluorescence). Di Sant'Agnese (1986) described bombesin-like-ir endocrine/paracrine cells in the human prostate, and hypothesized an important regulatory role of this peptide in prostatic physiology (and physiopathology), through effects on secretions and/or smooth muscle contraction. Considering that GRP-ir nerve terminals are present in close contact with smooth musculature of the canalicular organs and in a subepithelial localization in the glandular complexes, we may also hypothesize an intervention in contraction as well as secretory activities. GRP-ir intraepithelial nerve fibers identified in various tracts of bovine urethra may be sensory terminals, whose origin is not likely to be in the urogenital tract. It is also possible that this peptide participates in the modulation of visceral autonomic reflexes, being synthesized and released by noradrenergic excitatory nerve terminals coming from the sympathetic ganglia.

Both met- and leu-enkephalin-ir nerves have a similar abundant and extensive distribution in the bovine urogenital apparatus. They have been localized in the muscular coat of epididymis, ductus deferens and its ampulla, as well as in relationship with the acinar

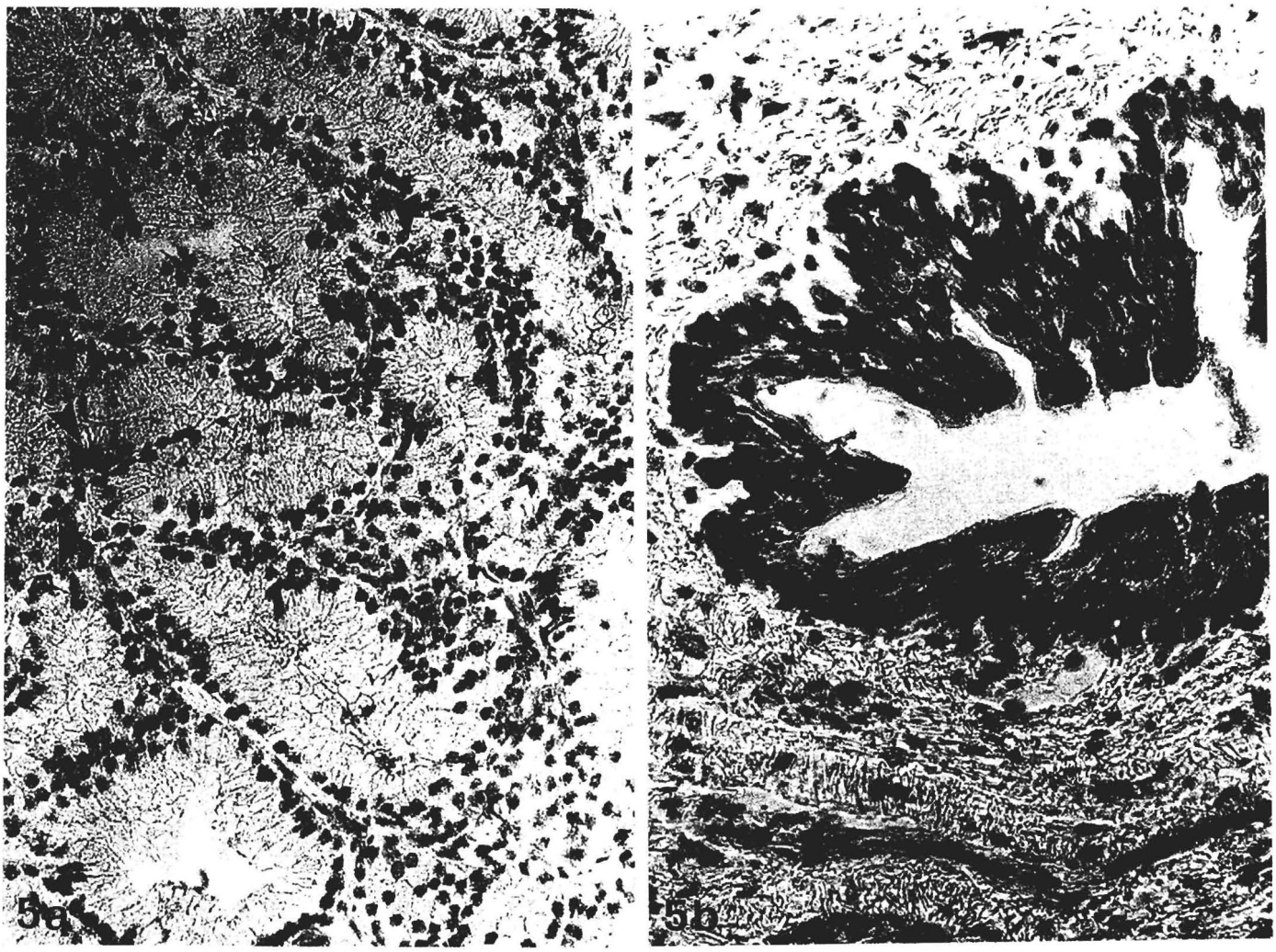


Fig. 5. 5-HT-like-immunoreactivity in epithelial endocrine cells. **a.** In the bulbourethral gland endocrine cells (arrowheads) are seen among the cells of the glandular epithelium. **b.** In the epithelium lining the penile urethra numerous 5-HT-ir endocrine cells are present among epithelial cells. They are slender and polymorphic. x 360

components of the prostatic complex and vesicular gland. It can be postulated that presence of enkephalins in the male genital organs could be linked to the control of smooth muscle, especially in those localizations such as the canalicular organs, where the progression of seminal fluid is promoted by muscular contractions. It has been hypothesized that excitatory adrenergic transmission is facilitated by substance P and inhibited by enkephalins in the rat ductus deferens, in such a way they modulate the process of ejaculation (Sastri et al., 1991). The present immunohistochemical data are in accordance with the biochemical findings by Sastry et al. (1991), who have identified these peptides in the bovine epididymis, vesicular gland and prostate, as well as in spermatozoa. A direct effect of them upon sperm motility may thus be hypothesized. Presence of Enk-ir nerves has been previously demonstrated in human *ductus deferens* muscular coat, prostatic stroma and vesicular gland smooth muscle (Vaalasti et al., 1986; Tainio, 1995). In *Equidae* (Arrighi and Domeneghini, 1997), enkephalin-like-ir nerve fibers were always immunolocalized in contact with smooth muscle components of some organs, such as epididymis, ductus deferens, its ampulla and vesicular gland; these are the same localizations in which enkephalin was seen in bovines, thus suggesting similar functional roles. Infrequent met-enkephalin-ir endocrine cells were detected in the bovine prostate. The presence of this type of endocrine cell was previously described only in the horse bulbourethral gland (Arrighi and Domeneghini, 1997). In these latter localizations, enkephalins may function as true endocrine or paracrine hormones, mediating or regulating overmentioned actions. Finally, the presence of neurons immunoreactive to GRP and enkephalins may suggest a possible local origin of some prostatic nerve terminals immunoreactive to these peptides.

Solitary CGRP-ir nerve fibers contacting the musculature of bovine prostatic complex and ductus deferens (with its ampullary portion) most likely originate from large fiber bundles peripherically located and have in turn a possible local origin from immunopositive neurons of intramural ganglia. They are probably implicated in motor functions facilitating the contractions of smooth muscle cells. A functional role towards modulation of the autonomic control of contractility of the smooth musculature cannot be excluded, as it has been demonstrated in other mammals (Afonso et al., 1996; Pinho et al., 1997). CGRP-ir nerve fibers seen in intraepithelial localization in the pelvic and penile urethra and others contacting the prostatic glandular epithelium could otherwise be sensory fibers and might have a function in pain transmission, as in other mammals (Iwanaga et al., 1985). CGRP implications in somato- and viscero-sensory systems are well known (Ishida-Yamamoto and Tohyama, 1989).

The presence of CGRP-ir nerve terminals which constitute motor end plates with some striated muscle of the bovine pelvic urethra is noteworthy, but not surprising. Evidence exists which supports the presence of this peptide in motor neurons innervating mammalian striated muscles (Kimura et al., 1994) and its release at mammalian neuromuscular junctions (Mora et al., 1989; Li and Dahlström, 1992), and also in visceral localizations (Rodrigo et al., 1994). In the motor neurons and nerves, CGRP is reputed to be released as a neuromodulator, which acts regulating the number of acetylcholine receptors (Ishida-Yamamoto and Tohyama, 1989; Csillik et al., 1993).

Substance P-like-ir is scarce in bovine male genital apparatus, and limited to extremely thin nerve fibers in the ductus deferens, prostate and bulbourethral gland. In addition, immunoreactive nerve fibers have been sporadically seen in perivascular localizations. The immunolocalization of Substance P, as well as that of CGRP, is limited in equine male genitalia, too (Arrighi and Domeneghini, 1997). Substance P-ir nerve fibers running close to smooth muscle cells in the canalicular ducts and glands may denote an implication in motor functions. In rats and men SP has been proven to facilitate adrenergic transmission, whereas enkephalins inhibited it (Sastri et al., 1991). On the other hand, sensory significances (Gu et al., 1983; Lamano Carvalho et al., 1986; Danuser et al., 1997; Kaleczyc et al., 1997) and/or a function in pain transmission, hypothesized in other peripheral localizations such as the gastrointestinal tract (Dolera et al., 1997), are hardly conceivable, as intraepithelial terminals were never noticed in bovine genitalia.

NPY-like-ir nerves were almost uniquely seen in contact with the smooth muscular coat of bovine ductus deferens. It is peculiar and noteworthy that, on the contrary, NPY was extensively utilized in innervation of equine male genitalia (Arrighi and Domeneghini, 1997). NPY-ir nerves are also the major peptide-containing neuronal component in male genitalia of man (Adrian et al., 1984; Tainio, 1995), guinea-pig and rat (Lamano Carvalho et al., 1986), species in which it is believed that it might be colocalized in noradrenaline-storing nerve terminals. In the pig it was recently demonstrated that NPY is colocalized with noradrenaline only in the perivasal fibers, whereas it may be a cholinergic modulator in the muscular coat of vas deferens (Kaleczyc et al., 1997). It is possible that in bovine male genital organs modulation of noradrenergic autonomic reflexes is in turn performed by the abundant GRP-releasing terminals. The presence of NPY-ir neurons in intramural ganglia might suggest that the prostatic nerve fibers utilizing this peptide are of local origin.

VIP-like-ir nerve fibers were seen almost exclusively in large bundles in the prostatic complex and, to a lesser extent, in the bulbourethral gland. Thus, in this species this peptide seems to have a minor importance in neuromodulation in comparison with male genitalia of other large animals such as *Equidae*, in which a massive VIP intervention in muscle relaxation, blood flow and secretion was hypothesized (Arrighi and Domeneghini, 1997). In porcine vas deferens (Kaleczyc

et al., 1997) non-noradrenergic (putative cholinergic) VIP-containing fibers were present in the lamina propria, which contrasts with our data on bovine organs.

As far as endocrine/paracrine cells are concerned, in the bovine urogenital apparatus we have demonstrated few met-enkephalin- and somatostatin-like ir cells in the prostate, whereas 5-HT-like-ir cells were frequent in the urethral epithelium and less numerous in the prostate and bulbourethral gland. In other species, endocrine cells have often been described: somatostatin-ir endocrine/paracrine cells are present in the human prostate (Di Sant'Agnese and De Mesy Jensen, 1984) and sheep urethro-prostatic complex (Vittoria et al., 1990). Serotonin-secreting cells have been immunohistochemically demonstrated along the urethral epithelia, in the male dog (Hanyu et al., 1987; Iwanaga et al., 1987), buffalo (Cecio and Vittoria, 1989) and sheep (Vittoria et al., 1990) and in the female horse, cattle, sheep and pig (Vittoria et al., 1992; Czaja et al., 1996). Calcitonin- and bombesin-like-ir cells were also described in the human prostate (Di Sant'Agnese, 1986). On the contrary, quite recently it was demonstrated that prostatic complexes of rat, guinea pig, cat and dog are devoid of endocrine cells (Angelsen et al., 1997). To our knowledge, presence of demonstrable endocrine/paracrine cells in the bulbourethral gland has been documented only in *Equidae* (Arrighi and Domeneghini, 1997), where a few number of epithelial cells are enkephalin-ir. Their possible functional role has been discussed above. In general, endocrine cells may play a local regulatory role in growth and differentiation as well as in the exocrine secretory process. Serotonin-secreting cells of urethra may play a role in monitoring chemical stimuli of the lumen, or may exert a paracrine influence over neighbouring cells, or, finally, realize a true endocrine secretion by their terminal ending on the basement membrane in vicinity of blood vessels. Endocrine cells of the prostate, whose distribution appears to be age-related in the human (Battaglia et al., 1994), may also be involved in the pathogenesis of prostatic cancer (Di Sant'Agnese and Cockett, 1994; Speights et al., 1994).

As a concluding remark, the finding that bovine urogenital apparatus is supplied by many peptidergic nerves, containing mainly GRP and enkephalins is noteworthy and might indicate that most of these peptides represent further neurotransmitters involved in motor and excitosecretory functions in the male genital tract, as well as in sensory attitudes. Nerve fibers utilizing GRP, enkephalins, CGRP and NPY could also have a local origin also, in addition to the extrinsic one. Comparison of the data obtained in this immunohistochemical study on the urogenital apparatus of *Bos taurus* with those obtained in Equidae (Arrighi and Domeneghini, 1997) shows a different utilization of the peptides tested in the various localizations. In both equine species it was evident that an extensive utilization of NPY exists, especially apparent in *Equus caballus*, and less evident in *Equus asinus*. Moreover, in equine genital apparatus NPY-ir fibers have been localized almost ubiquituously in contact with smooth muscle cells of arteries, and in this localization NPY is present even in the testis, where no other peptide was detected. On the contrary, in bovine urogenital apparatus it was possible to note a widespread distribution of GRP and enkephalins, while presence of NPY-ergic nerves was limited to few localizations, and was never observed in the artery wall. In the testis of this species, no peptides were detected. On the other hand, it has been recently stated that the testis has a limited capacity to autoregulate in other species, such as the rat, even if neuropeptides, for instance CGRP, may have potent vasodilator functions in testicular vasculature (Lissbrant et al., 1997).

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