Nerve Fibers in the Urethral Mucosa of Canine Penis are Immunoreactive for Both Substance P and Calcitonin Gene-Related Peptide

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Summary. The urethral mucosa in the canine penis was immunostained by use of antibodies against substance P and calcitonin gene-related peptide. Numerous nerve fibers immunoreactive for both peptides were found to invade the epithelium from the tunica propria; here they ran various courses with occasional branchings and conspicuous varicosities. Some looped to return into the tunica propria, while others ended on the epithelial surface. These findings confirm and extend our knowledge on the sensory innervation of the urethra which has previously been based on silver impregnation techniques.

Early silver-impregnation studies showed that the urethral mucosa in several mammals, including man, was rich in nervous elements (Seto, 1939; Mori, 1955; Nishio, 1960). Impregnated nerve fibers were reported to run complex, branching courses within the urethral epithelium (Nishio, 1960). In these studies, the urethral nerves were regarded as sensory nerves on the basis of their ending patterns. In fact, the urethra is known to produce a subtle sensation during urination, not to mention the characteristic pain during cannulation and urethritis. No information, however, is available on the nature of these nerves or the substances contained in them.

Recent advances in biochemical studies have revealed the presence and chemical structure of numerous bioactive peptides, most of which are isolated from the brain and gut. The production of antibodies against these "brain-gut peptides" allowed us to selectively stain peptide-containing cells distributed not only in the brain and gut, but throughout the nervous and endocrine systems of the body. Immunohistochemical studies have now encompassed the urogenital organs, revealing a rich supply of peptidergic nerves (Larsson et al., 1977; Alm et al., 1980; Gu et al., 1983). As far as we are aware, however, the urethra remains to be explored by immunohistochemistry.

In our preliminary survey on peptidergic neurons in the canine penis, we recognized that the urethral epithelium contained numerous nerve fibers which were positively stained with antibodies against substance P and calcitonin gene-related peptide (CGRP). Substance P is an undecapeptide known to be a transmitter of primary afferent neurons. It is also generally believed that substance P is a pain transmitter (Henry, 1980). Substance P is released in the dorsal horn of the spinal cord from the central terminals of certain primary afferent neurons (Otsuka and Konishi, 1983). Their
peripheral terminals, also showing intense immunoreactivity for substance P, have been reported to be distributed extensively in many peripheral organs, i.e., in the gut, skin, lung, adrenal gland and urogenital tract (Hökfelt et al., 1977; Sundler et al., 1977; Wharton et al., 1979; Gu et al., 1983; Kuramoto et al., 1985).

CGRP is a novel neuropeptide composed of 37 amino acid residues, whose presence has been revealed by using recombinant DNA and molecular biology techniques (Amara et al., 1982; Rosenfeld et al., 1983). Immunohistochemical studies by use of CGRP antisera have demonstrated an extensive localization of CGRP-containing neurons in both central and peripheral nervous systems (Rosenfeld et al., 1983). One predominant localization of CGRP neurons has been found in the sensory nerves, such as the small neurons in the trigeminal ganglion and in the spinal ganglia, as well as their terminals in the brain stem and spinal cord. Further immunohistochemical studies have reported the co-localization of CGRP and substance P in a single cell of the trigeminal ganglion and the dorsal root ganglia (Gibson et al., 1984; Lee et al., 1985; Terenghi et al., 1985). These findings strongly suggest that CGRP is also involved in pain sensation.

The present study reports on the frequent occurrence and peculiar structure of substance P/CGRP-containing nerves in the urethral mucosa, especially within the epithelium, in the canine penis.

MATERIALS AND METHODS

An antiserum against substance P (R 2404) was raised in rabbits and shown to react with intact substance P and substance P 3–11, but not with substance P 5–11 (Yanaihara, unpublished data). A monoclonal substance P antibody (NC 1/34-HL, Sera-Lab, England) was raised in tissue culture from rat spleen hybridoma. This antibody reacts with the 5–8 C-terminal fragments as well as intact substance P. A CGRP antiserum (RPN. 1842, Amersham, England) was raised in rabbits using synthetic rat CGRP conjugated to bovine serum albumin. In addition, rabbit antiseras against vasoactive intestinal polypeptide (VIP) (R 502, Yanaihara et al., 1977), neuropeptide Y (NPY) (RPN. 1702, Amersham, England), Met-enkephalin-Arg-Gly-Leu (Met-EK-8) (R 0171, Nihei and Iwanaga, 1985) and serotonin (Nishiitsuji-Uwo et al., 1984) were employed.

Three adult male dogs were used in this study. The animals were anesthetized with pentobarbiturate and perfused through the abdominal aorta with Ringer’s solution and consecutively with 10% formalin in 0.1 M phosphate buffer, pH 7.4. The penis was removed and immersed in the same fixative for 12 hrs. The fixed tissues were immersed in 30% sucrose solution at 4°C overnight. They were rapidly frozen in liquid nitrogen and sectioned at a 20 μm thickness in a cryostat.

Cryostat sections were processed to the peroxidase-antiperoxidase (PAP) method (Sternberger, 1979). In this staining, the rabbit antisera against substance P (R 2404), CGRP (RPN. 1842), VIP (R 502), NPY (RPN. 1702), Met-EK-8 (R 0171) and serotonin were used at a dilution of 1:2,000 to 1:5,000. For checking the specificity of the immunoreaction, the antisera were preincubated with the antigens (10 μg/ml diluted antiserum) for 24 hrs at 4°C. The synthetic substance P used in the absorption test was obtained by one of the authors (N.Y.) and the synthetic rat CGRP was purchased from Peninsula Laboratories, U.S.A. The absorbed antisera did not show any immunoreactivity in the sections examined.
Double immunofluorescence staining

The CGRP antiserum used was the same as used in the PAP method, while for substance P antibody, the rat monoclonal antibody was employed. Cryostat sections were first incubated with the rabbit CGRP antiserum diluted at 1:400 for 2 hrs, followed by fluorescein isothiocyanate (FITC)-coupled porcine anti-rabbit IgG (Dakopatts, Denmark) diluted at 1:20. Immunostained sections were thoroughly washed in phosphate-buffered saline (0.9% NaCl in 0.01 M sodium phosphate buffer, pH 7.2) and then incubated with the rat monoclonal antibody against substance P diluted at 1:100 for 2 hrs. The site of antigen-antibody reaction was revealed by rhodamine-labelled goat anti-rat IgG (Cappel, U.S.A.) diluted at 1:20.

Double-stained sections were observed and photographed under a Leiz Ortholux equipped with a fluorescence vertical illuminator (Ploemopak 2.2). Filter blocks L2.1 and N2.1 were used to view FITC and rhodamine fluorescence, respectively. With this combination of filters, each fluorophore could be viewed independently in the double-stained tissues. The specificity of the immunostaining was confirmed as follows: for the substance P antibody, when a rat normal serum or antigen-absorbed antibody was substituted, only FITC-fluorescent nerve fibers were recognized. On the other hand, when instead of the CGRP antiserum, a rabbit normal serum or antigen-absorbed antiserum was used, only rhodamine-fluorescent nerve fibers were present.

RESULTS

In the urethral mucosa of the canine penis, numerous nerve fibers in the tunica propria mucosae and within the epithelium were immunoreactive in the PAP method to both substance P and CGRP antisera. No nerves were stained positively with antisera

![Fig. 1. A single section from the urethra of canine penis double-stained by use of antibodies against CGRP and substance P. A nerve fiber takes a complex pathway within the epithelium showing FITC fluorescence for CGRP (a) and rhodamine fluorescence for substance P (b). The immunostained nerve fiber turns back and forms a loop (arrow) in the running course. ×480](image)
against VIP, NPY, Met-EK-8 or serotonin. The substance P- and CGRP-immunoreactive nerve fibers appeared to display similar localizations and running patterns, suggesting that substance P and CGRP were co-localized in a single neuron. This idea was confirmed by means of double immunofluorescence staining using the rabbit

Fig. 2. CGRP-immunoreactive nerve fibers in the urethral mucosa of the canine penis. PAP method. Three positive nerve fibers run toward the urethral lumen. One positive fiber indicated by an arrow appears to reach the lumen with a bulbous ending. ×480

Fig. 3. Two intraepithelial nerve fibers intensely immunoreactive for CGRP. The positive fibers extend very close to the lumen and end with a small swelling. ×875

Fig. 4. Two undulating nerve fibers showing CGRP immunoreactivity run toward the urethral lumen, and turn back toward the basement membrane. ×395
antiserum against CGRP (FITC) and the rat antibody against substance P (rhodamine) (Fig. 1).

The substance P/CGRP-immunoreactive nerve fibers that approached the urethral epithelium often ran transversely along the basement membrane of the epithelium,
before entering the epithelium. The immunoreactive nerves within the epithelium were thicker and more varicose than those in the tunica propria. The intraepithelial positive nerves showed various running patterns, which could be classified into the following four characteristic types: 1) A nerve fiber which took an almost straight course toward the mucosal surface and reached the lumen, frequently showing a bulbous ending there (Fig. 2, 3, 6); branching of this climbing nerve fiber was rare. This type of nerve tended to gather within the epithelium (Fig. 5). 2) An undulating nerve fiber which entered the epithelium ran toward the mucosal surface, looped back upon contacting the lumen or shortly before reaching the lumen, and returned toward the propria mucosae (Fig. 1, 4). It was not clear whether such a nerve should be judged as terminating within the epithelium or in the subepithelial layer. 3) The third type of immunoreactive fiber was characterized by a large terminal swelling within the epithelium (Fig. 5, 6). The size of the swellings varied, the largest ones corresponding to the size of nuclei of the epithelial cells. 4) In the fourth type, a nerve fiber took a convoluted pathway within the epithelium. Such a complexity was due to the formation of multiple loops, bifurcations and windings (Fig. 7). Occasionally, a complex termination displayed a loose glomerular appearance. Among these four types of the substance P/CGRP-positive nerve fibers, the first type was most numerous, while the third type was rarest. There were many cases of intermediate or mixed types between the four patterns described above.

The immunoreactive nerve fibers were more numerous in the proximal portion than in the distal portion of the pars cavernosa urethrae. Although positive nerves were recognized throughout the whole round of the epithelium in the cross-sectioned urethra, they tended to be more numerous in the mucous folds (Fig. 5, 6).

Further immunohistochemical staining showed that numerous flask- or spindle-shaped cells scattered within the epithelium were immunoreactive for serotonin. Most of the antisera against the bioactive peptides failed to stain the urethral endocrine-like cells, although only a few endocrine-like cells were found to react to the CGRP antiserum.

**DISCUSSION**

The present study revealed that numerous nerve fibers in the urethral mucosa of the canine penis were immunoreactive for both substance P and CGRP. Although a rich innervation in the urethral epithelium had been depicted by earlier silver-impregnation studies (Seto, 1939; Mori, 1955; Nishio, 1960), we were able to demonstrate more clearly the distribution and the running patterns of the urethral nerves by use of immunohistochemical techniques. Thus, the existence of nerves open to the urethral lumen and those with a large terminal swelling have been newly described in the present study.

Furthermore, this study has confirmed the idea that substance P and CGRP were co-localized in a single neuron (Gibson et al., 1984; Lee et al., 1985; Terenghi et al., 1985). No nerve fibers in the urethral epithelium were found to be immunoreactive for serotonin or other neuropeptides including VIP, NPY and Met-EK-8. Therefore, the urethral epithelium can be said to be innervated predominantly, if not exclusively, by the substance P/CGRP-containing neurons.

The present immunohistochemical demonstration of substance P in the urethral nerves supports the widely accepted hypothesis that they are of a sensory nature.
The substance P/CGRP-containing nerves appear to be responsible for the subtle, tactile-like sensation or characteristic pain perceived in the urethra. The substance P/CGRP-positive nerves directly reaching the surface of epithelium seem to be at a favorable position for receiving various kinds of stimuli from the lumen. As for the nerves isolated from the urethral lumen, especially those showing a complex termination and glomerular structure, the possibility is proposed that they may react to mechanical stimuli according to the extension or contraction of the urethral wall.

On the other hand, several researchers have reported the existence of chromaffin cells in the urethral epithelium by use of fluorescence histochemistry and electron microscopy (Dixon et al., 1973; di Sant’Agnese and de Mesy Jensen, 1984). These flask-shaped chromaffin cells were reported to contain electron-dense secretory granules in the basal cytoplasm and to reach the lumen, their apical cytoplasm covered by a tuft of microvilli. As in the case of the endocrine cells in the gut (Fujita and Kobayashi, 1973), these urethral chromaffin cells are thought to recognize chemical information from the urethral lumen and release their secretions in response to it. The present study first demonstrated the presence of immunoreactivities for serotonin and CGRP in the urethral chromaffin cells. In addition to the possible sensory neurons containing substance P and CGRP, the urethral chromaffin cells appear to play an important role in regulating the functions of the urethra.

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