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Immunohistochemical Demonstration of Nerves Containing Protein Gene Product 9.5 (PGP 9.5) and Neuropeptides in the Mouse Kidney

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Abstract The immunoreactivity for protein gene product (PGP) 9.5, a neuronal marker, was examined in the mouse kidney. Immunoreactivities for neuropeptide Y (NPY), calcitonin gene-related peptide (CGRP), substance P (SP) and vasoactive intestinal polypeptide (VIP) were also studied. Nerve fibers with PGP 9.5 immunoreactivity were widely recognized in all segments of the renal vascular system, i.e., in the interlobar, arcuate, and interlobular arteries and veins, in the afferent and efferent arterioles, and in the vasa recta of the outer stripe of the outer zone of the medulla. In the renal tubules, there were also a few varicose terminals showing PGP 9.5 immunoreactivity. NPY-immunoreactive fibers occurred widely in the renal vascular system forming a plexus. Moderate number of CGRP-immunoreactive fibers and a few SP-immunoreactive fibers, which seemed to be co-localized partly, were distributed mainly in the interlobar and arcuate arteries. No VIP-positive nerves were recognizable. Each positive type of nerve fibers showed an unique distribution and seemed to act differently in the various branches of the renal vascular system.

Key words : Protein gene product 9.5-Neuropeptide Y-Calcitonin gene-related peptide-Substance P-Kidney innervation

Introduction

Protein gene product (PGP) 9.5, which was discovered by Jackson and Thompson[1] in 1981, is ubiquitin carboxyl-terminal hydrase and is now used as a general neuronal marker.

Immunohistochemistry for PGP 9.5 seemed to reveal all types of motor, sensory, and autonomic nerve fibers[2]. Gulbenkian et al.[2] reported that, at the electron microscopical level, PGP 9.5 immunoreactivity was found in the axonal cytoplasm and did not appear to be associated with cytoskeletal elements or vesicular structures. In comparison to neurofilament protein (NFP) and neuron specific enolase (NSE), which are more universally applicable neuronal markers, PGP 9.5 seems to be a more sensitive general neuronal marker for peripheral innervation patterns because of its discrimination of more terminal axonal structures[3]. PGP 9.5 immunoreactivity in the kidney was found in the juxtaglomerular apparatus and in the cells of parts of the distal tubules (P. Barber, unpublished data), but in the mouse kidney, no one has observed PGP 9.5 immunoreactive nerves or revealed its distribution.

Renal peptidergic innervation has been discussed by various researchers using a number of mammalian species, though the rat

has been used particularly frequently. Knight et al.[4,5], Barajas et al.[6], and Reinecke and Forssmann[7] have worked with neuropeptide Y (NPY) ; Knight et al.[5,8], Barajas et al.[6,9], Reinecke and Forssmann[7], and Geppetti et al.[10] with calcitonin gene-related peptide (CGRP) ; Knight et al.[8,11], Ferguson and Bell[12], Reinecke and Forssmann[7], and Barajas and Liu[6] with substance P in the rat kidney ; and Barajas et al.[13], Reinecke and Forssmann[7], and Knight et al.[4,14] with vasoactive intestinal polypeptide (VIP). Although Dieterich[15] described the innervation of the rat kidney with electron microscopy, no one has observed the whole renal innervation yet with light microscopy. Therefore, in the present study the distribution and density of PGP 9.5 immunoreactive nerve fibers and bundles are described in order to express the whole renal innervation, and they are compared with the distribution and density of NPY, CGRP, SP and VIP immunoreactivity, because these peptides are thought to perform renal neuromodulatory functions.

Materials and Methods

Animals and tissue preparation

Ten ICR adult mice of either sex weighing 33-48 g and aged 8-10 weeks were anesthetized with an intraperitoneal injection of pentobarbital (60mg/kg) and perfused through the left ventricle with physiological saline and subsequently with Zamboni solution[16]. After perfusion, the kidney with the upper ureter were dissected out and postfixed by immersion in the same fixative for an additional 8 h. They were then immersed in 0.1M phosphate buffer containing 30% sucrose for at least 48h. Tissue blocks were cut horizontally at 40μm thick on a cryostat. Serial sections were collected on gelatin-coated slides and allowed to dry for 60 min at room temperature. Then the slides were washed three times in 0.02M phosphate-buffered saline (PBS).

Immunohistochemical procedure

Immunostaining was performed by the avidin-biotin peroxidase (AB) method. The sections were incubated with rabbit polyclonal antibodies to PGP 9.5 (diluted 1 : 2500 ; Ultraclone), CGRP (1 : 2500 ; Cambridge Research Biomedical), NPY (1 : 2500 ; Cambridge Research Biomedical), VIP (1 : 2500 ; Cambridge Research Biomedical) and with rat monoclonal antibodies to SP (1 : 2500 ; Chemicon) sepaparately, diluted in a solution of 1% normal goat serum in 0.02M phosphate -buffered saline, for 72h in a humid atomosphere at 4℃.

For primary antisera raised in rabbits, the second antibody was goat biotinylated anti-rabbit IgG (Dakopatts), and for primary antisera raised in rats, rabbit biotinylated anti -rat IgG (Dakopatts) was used. Sections were then incubated with the IgG antibodies for 24 h and streptavidin-horseradish peroxidase (Vector) for 12h at 4℃. The immunohistochemical reaction product was developed by incubation of the sections in a solution of 0.05 % diaminobenzidine hydrochloride (Sigma, St.Louis), 6% nickel ammonium sulphate, and 0.01% hydrogen peroxide in 0.05M Tris buffer for 15-20 min. Control sections were incubated with either 0.02M PBS (PGP 9.5) or antiserum pretreated with an excess of antigens (10 nmol/ml ; SP, CGRP and NPY). These reactions were completely blocked. Some of the immunostained cryostat sections were counter stained with cresyl-violet. After being washed, tissue sections were dehydrated in a graded series of alcohol, and mounted under coverslips with Permount.

Quantitative analysis

The innervation was described according to the renal vascular segments, i.e. (1) the interlobar arteries and veins, (2) the arcuate arteries and veins, (3) the interlobular arteries and veins, (4) the afferent and efferent glomerular arteries, (5) the medullary vascular bundles, and (6) tubules and collecting tubules. The number of positive nerve fibers in each segment of the kidney was assessed visually and allocated to one of six categories, absent(−), very few(+/−), few (+), moderate(++), many(+++) and abundant(++++).

Results

PGP 9.5 immunohistochemistry

Examination of PGP 9.5 immunoreactivity in the mouse kidney revealed a rich nerve

Table. Relative number of PGY, CGRP, and SP-immunoreactive nerves in the mouse kidney

	PGP9.5	NPY	CGRP	SP
Interlobar arteries	++++	++++	+++	++
Arcuate arteries	+++	+++	++	+
Interlobular arteries	+++	+++	+	+/−
Afferent glomerular arteries	+	+	+/−	−※
Efferent glomerular arteries	+	+	+/−	−
Interlobar veins	++	++	+	+/−
Arcuate veins	+	+	+/−	−
Interlobular veins	+/−	+/−	−	−
Medullar arteries	+	+	−	−
Tubules/ collecting tubules	+/−	+/−	−	−

Relative numbers of immunoreactive fibers are graded as follows : absent (−), very few (+/−), few (+), moderate (++), many (+++) and abundant (++++)

* a few positive nerve fibers were observed in the afferent glomerular arteries which directly branched from the arcuate arteries.

supply, forming a mesh-like pattern around the arterial system (table). There was an abundant nerve plexus of varicose axons innervating the circumference of the interlobar, arcuate and interlobular arteries (Figs. 1 A, B and C). Nerve bundles with PGP 9.5 immunoreactivity was also observed in the interlobar, arcuate, and interlobular arteries (Figs. 1 A, B and C). Dense network of varicose fibers was also seen around the afferent and efferent arterioles (Fig. 1 D). In the segments of the renal vein, nerve fibers which formed a thin perivascular nerve plexus were seen at the interlobar, arcuate and interlobular veins. Few PGP 9.5 immunoreactive nerve fibers were observed in the medulla, but nerve fibers in the efferent arterioles of the juxtamedullary cortical regions extended to the vasa recta of the outer stripe of the outer zone of the medulla (Figs. 1 E and F). In the renal tubules, there were a few varicose terminals with PGP 9.5 immunoreactivity, but they were rarely found in the peritubular interstitum. In the mouse kidney PGP 9.5 immunoreactivity could not be found in the juxtaglomerular apparatus or in the cells of the distal tubules.

Density and distibution of peptide-containing nerves

The distribution patterns of neuropeptide-containing nerves were all dissimilar in the

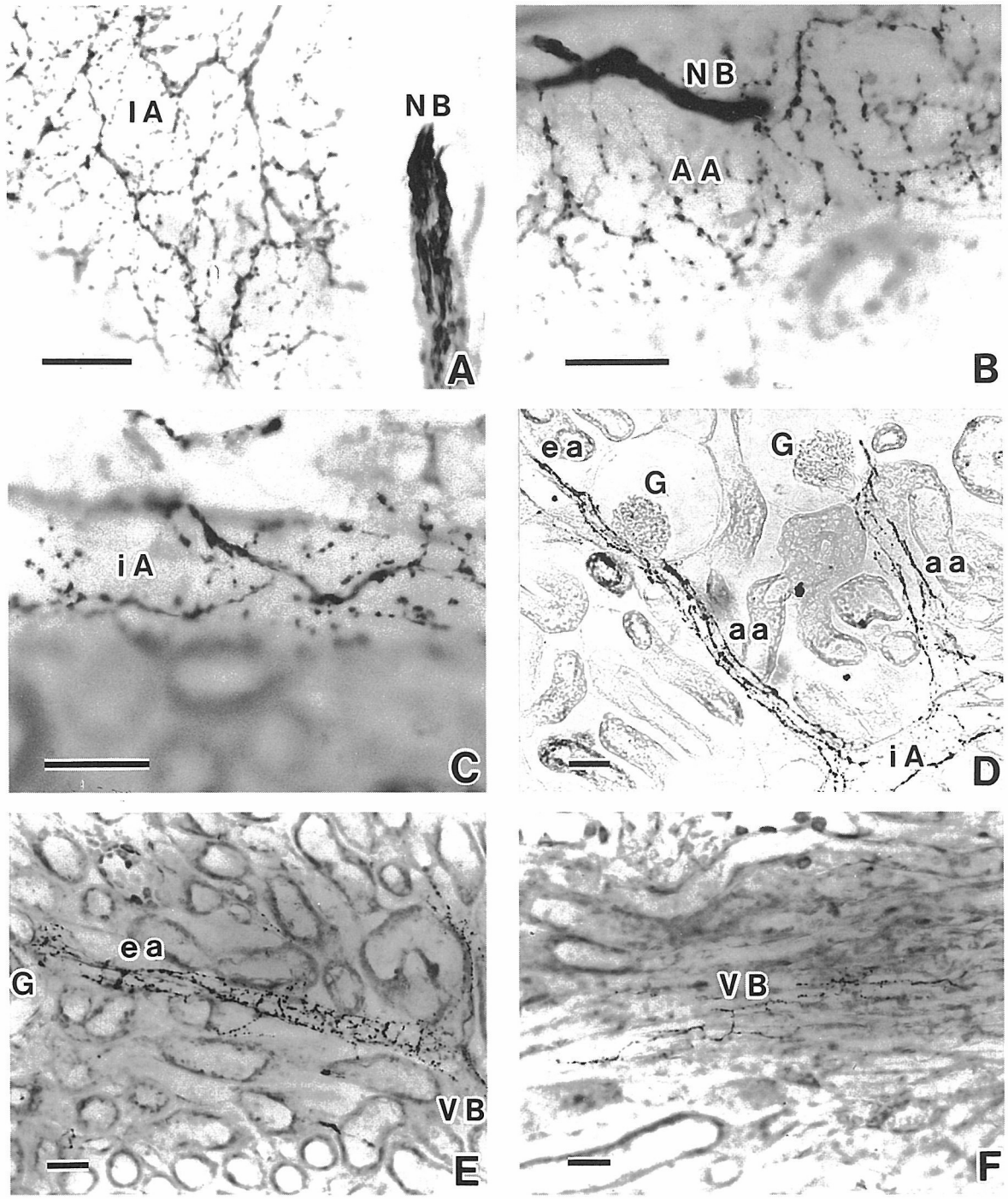

Fig. 1. PGP 9.5-immunoreactive nerves in the mouse kidney. **A**, **B**, and **C** Interlobar, arcuate and interlobular arteries with a plexus of varicosities and nerve bundles, respectively. **D** A PGP 9.5-immunoreactive nerve plexus innervating an afferent arteriole branching from the interlobular artery and an efferent arteriole. **E** PGP 9.5-immunoreactive varicose fibers extend from an efferent arteriole at the juxtamedullary region to a medullary vascular bundle in the outer stripe of the outer zone. **F** PGP 9.5-immunoreactive nerve terminals at the vascular bundle of the outer stripe of the outer zone of the medulla. IA : interlobar artery ; AA : arcuate artery ; iA : interlobular artery ; aa : afferent arteriole ; ea : efferent arteriole ; NB : nerve bundle ; VB : vascular bundle ; and G : glomerulus. Scale Bar : 20μm

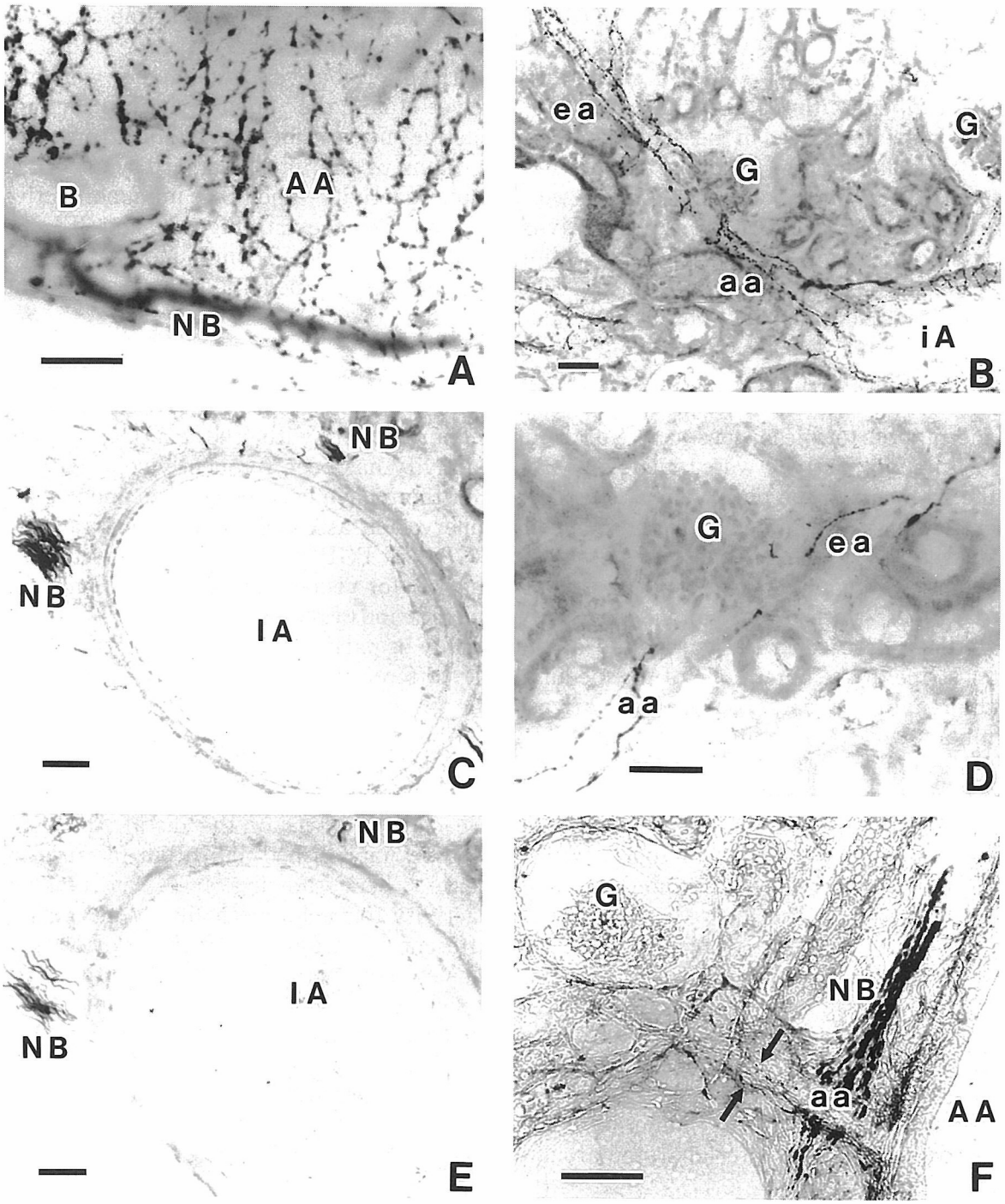

Fig. 2. NPY-(A and B), CGRP-(C and D) and SP-immunoreactive(E and F) nerve fibers and nerve bundles in the mouse kidney. A NPY-immunoreactive nerve plexus on an arcuate artery. B an interlobular artery, and afferent and efferent arterioles with a plexus of NPY-immunoreactive varicose fibers. C, E CGRP-and SP-immunoreactive nerve fibers in the same nerve bundles of an interlobar artery in serial sections. D CGRP-immunoreactive fibers on an afferent and an efferent arteriole. F SP-immunoreactive fibers on an afferent arteriole which directly branches from the arcuate artery (arrow). IA : interlobar artery ; AA : arcuate artery ; iA : interlobular artery ; aa : afferent arteriole ; ea : efferent arteriole ; B : small branch ; NB : nerve bundle ; and G : glomerulus. Scale Bar : 20μm

renal vascular system. There were quantitative differences (Table) and each neuropeptide had a characteristic innervation pattern.

Nerve fibers with NPY immunoreactivity were widely distributed in the segments of the renal vascular system, forming a mesh-like pattern. NPY-immunoreactive fibers were distributed widely and richly in the perivasculature and were similar to PGP 9.5 immunoreactivity in distribution pattern and quantity. In the interlobar, the arcuate, and the interlobular arteries, there were a few nerve bundles with NPY immunoreactivity (Fig. 2A). Around the afferent and efferent glomerular arterioles, dense networks of NPY immunoreactivity were found (Fig. 2B). In the segments of the renal vein, a few nerve fibers with NPY immunoreactivity, which formed a nerve plexus, were seen at the interlobar, arcuate, and interlobular veins. Nerve fibers with NPY immunoreactivity were also observed in the vasa recta of the outer stripe of the outer zone of the medulla. In the renal tubules, there were few varicose terminals with NPY immunoreactivity.

The number of CGRP-immunoreactive fibers was less than that of PGP 9.5-immunoreactive and NPY-immunoreactive fibers. Nerve fibers with CGRP immunoreactivity were mainly observed at the interlobar and arcuate arteries. The nerve bundles with CGRP immunoreactivity were also seen at the interlobar and arcuate arteries (Fig. 2C). But there was only a small number of CGRP-immunoreactive fibers on the interlobular arteries and the glomerular arterioles (Fig. 2 D). In the segments of the renal vein, a very small number of CGRP-immunoreactive fibers was seen at the interlobar and arcuate veins.

SP-immunoreactive fibers were mainly distributed in the interlobar and arcuate arteries, though they were more sparse than NPY- and CGRP-immunoreactive fibers. SP -immunoreactive nerve bundles were seen in the interlobar and arcuate arteries (Fig.2E). In the interlobular arteries and the vicinity of vascular pole, there were few SP-immunoreactive fibers, but a few were observed in the afferent glomerular arterioles which directly branched from the arcuate arteries (Fig. 2F). There were none in the efferent glomerular arterioles. In the segments of the renal vein, a few SP-immunoreactive nerve fibers were on the interlobar vein, though they were much fewer than the other peptides-immunoreactive nerve fibers. Judging from serial sections of the interlobar artery, CGRP immunoreactivity and SP immunoreactivity appeared to coexist (Figs. 2C and 2E).

There were no VIP-immunoreactive fibers in the cortex or medulla of the mouse kidney.

Discussion

Recently papers comparing PGP 9.5 immunoreactivity with other peptides immunoreactivity has been frequently reported[2,18–24]. But there have been no reports about kidney innervation assessed by immunostaining for PGP 9.5. PGP 9.5 seems to be a reliable marker for visualizing the general pattern of innervation of the cardiovascular system, and it may be particularly useful when examining the differential density of nerve subtypes and the changes in cardiovascular innervation during development, aging, and disease[2].

PGP 9.5 immunoreactivity has been reported in the juxtaglomerular apparatus and in the cells of parts of the distal tubules (P. Barber, unpublished data), but in our study, nerve fibers showing PGP 9.5 immunoreactivity formed a mesh-like pattern around afferent and efferent glomerular arteries and was not confirmed particularly to the juxtaglomerular apparatus or the cells of parts of the distal tubules.

In the present study the distribution of PGP 9.5 immunoreactivity resembled the pattern of innervation in the rat kidney presented by Dieterich[15], although the author did not describe the innervation of the renal venous system. Nerve fibers and bundles of PGP 9.5 immunoreactivity were distributed in the interlobar, arcuate, and interlobular arteries and veins, in the afferent and efferent arterioles, and in the vasa recta of the outer stripe of the outer zone of the medulla. In the renal tubules, there were a few varicose terminals of PGP 9.5-immunoreactivity. In addition to mice, we have found that PGP 9.5 immunoreactivity is well expressed in the renal vascular innervation of man (unpublished data).

The present study also demonstrated inner-

vation of the mouse kidney by use of some neuropeptides.

Many NPY-immunoreactive fibers were observed and NPY was found to be the most abundant neuropeptide. NPY-immunoreactive nerves are generally associated with noradrenergic nerves and have a vasoconstrictive function[24], but they also occur in non-catecholaminergic nerves[21,22,25]. In the kidney, NPY nerves, which are associated with cathecolaminergic nerves, may be of sympathetic origin and have vasoconstrictive effects[7]. The result of double immunolabeling of DBH (dopamine beta-hydroxylase) and NPY suggested that all NPY intrarenal nerves were noradrenergic[4].

The CGRP-containing fibers may represent dual afferent and efferent functions. Efferent CGRP nerves have a vasodilator function[26]. CGRP-immunoreactive fibers are distributed widely in the renal vascular systems, though they are fewer than PGP 9.5-immunoreactive and NPY-immunoreactive fibers. CGRP-immunoreactive nerves may regulate the renal blood flow by efferent vasodilator action. On the other hand, intrarenal afferent CGRP-immunoreactive nerves may have baroreceptive and chemoreceptive functions[8].

The SP-containig fibers in the rat kidney may transmit sensory information, which may represent baroreceptors and chemoreceptors[12]. Most perivascular SP-immunoreactive axons terminate on interlobar and arcuate arteries in the rat kidney[11]. In the mouse kidney, SP-immunoreactive nerve fibers are mainly distributed in the interlobar and arcuate arteries, similarly to those of the rat, though only a few positive nerve fibers were observed in the afferent glomerular arteries which directly branched from the arcuate arteries. The work by Knight et al.[11] and our results suggested that SP-immnoreactive nerves may transmit sensory information from large intrarenal arteries. The SP-immunoreactive axons comprised a subpopulation of the CGRP axon population in the rat kidney and there was no evidence for a separate population of SP-immunoreactive axons[8]. All renal afferent somata immunoreactive for SP throughout the dorsal root ganglia also contain CGRP[27]. CGRP-immunoreactive and SP-immunoreactive fibers may also coexist in the interlobar and arcuate artery of the mouse kidney. These two peptides might have cooperative function in the renal afferent system.

VIP-immunoreactive nerves have been found in the kidneys of the pig, dog, and rat[4,7,14,15]. Knight et al.[4] demonstrated that VIP-containing fibers supplying the arcuate and interlobular vessels are noradrenergic and selectively dilate certain parts of the arterial tree. In view of the results using the guinea pig, rat, cat, dog, pig, and tree shrew, intrarenal distribution of VIP-immunoreactive nerves was different among species[28]. In our study, VIP-immunoreactive nerves were not found in the mouse kidney. Larsson et al.[29] have also reported that VIP-immunoreactive nerves were not observed in the mouse kidney.

In the present study, when comparing with PGP9.5-immunoreactive fibers in the mouse kidney, NPY-immunoreactive fibers were most numerous, and CGRP-immunoreactive fibers were more numerous than SP-immunoreactive fibers. Thus PGP 9.5 seems to be a reliable marker for visualizing the general pattern of innervation of the kidney and may be particularly useful when examining the differential density of nerve subtypes and the changes in kidney innervation of certain renal disease. Further studies on the poorly understood relation between renal function and renal innervation may be useful for the treatment of renal disease and the long-term viability of renal transplants.

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