**GABA-immunoreactive Structures in Rat Kidney**

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We examined the distribution of gamma-aminobutyric acid-like immunoreactivity (GABA-LI) in the rat kidney by light and electron microscopy. In vibratome sections, GABA-LI was present in both the renal medulla and cortex. The inner stripe of the outer medulla was most heavily and almost homogeneously labeled, whereas GABA-LI in the cortex was mainly confined only to some tubules. GABA-positive structures involved the epithelial cells of the thin and the thick ascending limbs of the loop of Henle, the collecting tubules, and the collecting ducts. In GABA-positive connecting tubules and collecting ducts the immunoreactivity was present in the cytoplasm of about half of the epithelial cells. As revealed by electron microscopy, the labeled cells in the collecting tubules were the light (principal) cells. No GABA-LI occurred in neuronal structures. These findings are consistent with the presence of a non-neuronal GABA system in the rat kidney. Furthermore, the specific distribution of GABA in the tubular epithelium suggests a functional significance of this amino acid in tubular transport processes.

**KEY WORDS:** Gamma-aminobutyric acid (GABA); Immunohistochemistry; Rat kidney; Renal tubules; Loop of Henle.

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**Original Article**

**Introduction**

Increasing evidence indicates that gamma-aminobutyric acid (GABA) not only is a transmitter in the vertebrate central and peripheral nervous systems but also mediates various functional responses in the kidney. These studies showed that GABA and the GABA-metabolic enzymes, i.e., glutamate decarboxylase (GAD) and GABA-transaminase (GABA-T), are concentrated in the tubular fraction of the renal cortex (9-10). However, GABA and its marker enzyme, GAD, have not been visualized in the mammalian kidney as yet. Since the cellular localization of GABA may be helpful in understanding its possible functional role, in the present study the distribution of GABA was examined in the rat kidney with immunohistochemical techniques, by using different polyclonal anti-GABA antisera.

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**Materials and Methods**

**Animals and Perfusion.** Adult male CD rats, weighing 300–350 g, were obtained from Charles River Wiga GmbH (Sulzfeld, FRG). Rats had free access to water and standard food. Animals were anesthetized with ether and transcardially perfused first with 150–200 ml PBS [phosphate buffered saline; 0.9% NaCl in a 10 mM phosphate buffer (PB), pH 7.4], then with 300–350 ml of a fixative containing 4% formaldehyde (FA) and 0.2% glutaraldehyde (GA), or 3% GA without FA in 0.12 M PB.

**Tissue Sections.** Both kidneys were sliced transversely at 4–5-mm thickness. Tissue blocks were washed in 0.1 M Tris-HCl-buffered saline, pH 7.6 (TBS) for 24 hr at 4°C and sectioned on a vibratome at 75 μm. For light microscopy, sections were pre-treated with methanolic 30% H2O2 for 10 min and washed thoroughly in TBS. For electron microscopy the vibratome sections were infiltrated with 30% sucrose in 0.05 M TB for 1-2 hr, then frozen at -4°C and thawed before application for GABA immunocytochemistry.

**Pre-embedding Immunocytochemistry.** Incubations were carried out at 37°C in the following sequence: (a) in normal goat serum (Sebak; Aidienbach, FRG) for 1 hr; (b) in anti-GABA serum No.483a (2) or No.7 diluted 1:1000 to 1:9000 and 1:2000 to 1:8000, respectively, in a solution containing 20% normal goat serum and 10% normal rat serum (GRS) for 10 to 16 hr; (c) goat anti-rabbit IgG (Jackson Immunoresearch Laboratories; West Grove, PA) diluted in GRS for 1–1.5 hr; (d) rabbit PAP (Jackson) diluted 1:400 in GRS for 1–1.5 hr (22). Sites of immunoreactions were visualized by the peroxidase–anti-peroxidase (PAP) technique applying 3,3′-diaminobenzidine (0.05%); Sigma, St Louis, MO) in the presence of NiCl2,6H2O (0.1%) and H2O2 (0.005%) for 10–12 min. After each incubation step, sec-

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tions were washed three times in 0.1 M TBS. Light micrographs were taken in a Zeiss photomicroscope.

For electron microscopy, samples were washed in 0.13 M PB after the DAB reaction, post-fixed in phosphate-buffered 1% OsO4 for 1 hr, dehydrated in alcohol, and embedded flat in Araldite (Durcupan ACM; Fluka, Buchs, Switzerland). Ultra-thin sections were examined with Zeiss EM10C and EM902 electron microscopes.

Post-embedding Immunocytochemistry. The frozen and thawed vibratome sections were osmicated, dehydrated, and embedded in Durcupan ACM. Semi-thin sections (1–2 μm) were cut and air-dried onto glass slides. The sections were etched according to Maxwell's method (16) and the osmium was removed with NaIO4 (21). Immunoreagents were overlaided onto the sections in the order mentioned earlier, i.e., the immunocytochemical protocol was the same as in the pre-embedding procedure. The reaction was performed with the GABA antibody No. 7, diluted 1:2000 to 1:6000.

The specificity of the method and of the antibodies was tested (a) by replacement of the primary antibody with normal rabbit serum or (b) by pre-absorption of primary antisera using GABA (Sigma) conjugated to bovine serum albumin (BSA) via GA (13,20). In sections of both types of control experiments no immunostaining was observed.

Antisera. Two different antisera to GABA were employed. They were prepared by Drs. P. Petrusz and P. Somogyi and designated as No.483a (2) and No.7, respectively. These antisera had been found to be highly specific for GABA conjugated to tissue proteins via glutaraldehyde (13,14,20) and gave identical results in our experiments. As the tissue specificity of the antisera is very important for the correct interpretation of our results, further control experiments were performed to exclude any crossreactivity. The primary antisera were pre-absorbed with glutaraldehyde-BSA and β-alanine-glutaraldehyde-BSA conjugates, respectively. In these cases the overall staining intensity was slightly reduced but no selective loss of immunoreactivity was observed.

Results

At low magnification, GABA-LI was present in both the medulla and the cortex of the kidney. The inner stripe of the outer medulla displayed a very intense, homogeneous staining, whereas in the outer stripe, inner medulla, and cortex immunoreaction was restricted to some tubules (Figure 1). Occasionally the glomeruli appeared to display weak staining, but control experiments with GABA-pre-absorbed primary antisera clearly indicated that this staining is not specific.

In the cortex, at higher magnification, the GABA-LI proved to be present in the connecting tubules and in all collecting ducts (Figure 2). The walls of these tubules exhibited a characteristic mosaic-like pattern, with about half of the epithelial cells being immunoreactive for GABA whereas the other cells were negative.

The strong immunostaining of medullary structures was further studied in semi-thin sections (Figures 3 and 4). The higher resolution obtained with the post-embedding method made it possible to reveal that in the medulla the collecting tubules were labeled. The majority of the tubular epithelial cells were GABA immunopositive; another fraction of them, and most other (non-tubular) cells, were clearly devoid of reaction. GABA-positive neuronal elements were not seen in the kidney.

In agreement with the light microscopic findings, electron microscopic examinations revealed no GABA immunoreaction in renal corpuscles and in the proximal convoluted tubules. However, the reaction product was clearly seen in the thin limb of Henle's loop (Figure 5). Despite the fact that the squamous epithelial cells of these tubules appear identical from a fine-structural point of view, GABA immunoreactivity was present only in about half of them. The GABA-positive cells were evenly distributed; practically every second one contained electron-dense PAP reaction product. Epithelial cells of the distal tubules (Figure 6) also showed the same type of GABA immunoreactivity pattern. The connecting tubules and collecting ducts consist of two types of epithelial cell: (a) principal or "light" cells with few organelles and relatively smooth convex apical surface, and (b) intercalated or "dark" cells with more abundant organelles, denser cytoplasm, and apically situated long microvilli. As shown in Figure 7, the GABA-LI was exclusively present in one of these two cell types, i.e., those showing the features of principal cells. It must be mentioned that the labeling pattern of GABA-immunoreactive tubule cells both in the cortex and medulla was the same. The reaction product was evenly distributed in the cytoplasm and the nuclei were rarely and only faintly labeled. Very few non-tubule cells possessed GABA-LI. In several cases the capillary endothelium and fibroblasts were stained (Figure 8).

Discussion

The distribution of GABA-LI in the rat kidney is in line with earlier biochemical results showing that GABA and its synthesizing enzyme, GAD, are present in all fractions of rat renal homogenates, with the tubule fraction being particularly rich in both markers (9,10). It has been shown that in the kidney cortex GABA concentrations are highest in the juxtamedullary region (10) and that the activity of GABA synthesis via GAD is higher in the tubule than in the glomerular fraction of renal homogenates (9). According to recent HPLC measurements (6), the GABA content in the rat renal cortex and medulla was 19 ± 2 and 92 ± 7 nmol/wet weight, respectively (mean ± SD; n = 5). The immunocytochemical results presented here show very good correlation with these biochemical findings, because the medulla displayed very intense, homogeneous

Figure 1. Distribution of GABA-LI in a 70-μm thick section of the rat kidney. Note the heavy immunolabeling in the inner stripe of the outer medulla and the specific staining of collecting ducts in the outer stripe, as well as in several tubule structures of the cortex. Bar = 500 μm.

Figure 2. Renal cortex; GABA-LI in a connecting tube joining a collecting duct. Note that only about 50% of epithelial cells are GABA positive and form a chessboard-like pattern. Bar = 75 μm.

Figure 3. Light micrograph of a semi-thin section (1 μm) from the outer medulla. Post-embedding immunostaining reveals that only the tubule structures are labeled. Bar = 200 μm.

Figure 4. Higher magnification picture of the inner medulla showing clearly non-labeled cells in collecting tubules. Bar = 20 μm.
staining, whereas in the cortex the GABA-LI was present only in some tubules, the juxtamedullary region being more heavily labeled. GABA-immunoreactive cells showed a well-defined characteristic distribution, with no reaction product being found in any elements of the renal corpuscle and the proximal tubules. However, GABA-LI could be demonstrated in other parts of the nephron. It is interesting to note that in the GABA-positive connecting tubules and cortical collection ducts only about 50% of epithelial cells were immunostained. In fact, in the loops of Henle the epithelial cells are known to be morphologically identical. Therefore, the selective distribution of GABA in these cells does not correlate with a morphological heterogeneity. It is the subject of further studies to demonstrate if the presence of GABA in these cells reflects different biochemical machineries and/or different functional status.

In connecting tubules and collecting ducts the epithelium consists of two morphologically and biochemically well-defined cell populations of different developmental origin, i.e., principal and intercalated cells (17). In these tubules GABA-LI was confined exclusively to the principal cells. This observation was further supported by our recent finding that inhibition of the (Na,K)-ATPase, a marker enzyme of principal cells (19), evoked the release of endogenous GABA from slices of rat renal cortex and medulla (6).

The GABA content of the kidney may be derived from alternative sources. In addition to an intramural biosynthesis via GAD, GABA may also be taken up from extracellular fluids by uptake mechanisms. In fact, biochemical evidence has been presented that in preparations of the rat renal cortex active high- and low-affinity GABA uptake systems operate in vitro (11,18). GABA-T is considered to be a poor marker of GABAergic cells. Therefore, it is not expected to be consequently co-localized with its substrate, GABA. Although the enzyme seems to be predominantly present in the renal cortex (27), the GABA content, as demonstrated biochemically and immunohistochemically, is considerably higher in the medulla. In a preliminary study, Wu et al. (27) reported on GABA-T immunoreactivity in the renal cortex, in the apical portion of the proximal tubules (mainly in the brush border region), and in the distal convoluted tubule epithelium, where the cytoplasm but not the nucleus was labeled. This distribution profile corresponds poorly with our findings of the distribution of GABA-LI, because we did not find GABA-LI in proximal and distal convoluted tubules. This non-correspondence supports the view that medullary GABA content may be regulated by enzymes other than GABA-T and/or by GABA transport (uptake) mechanisms.

Although the role of GABA in the kidney is not yet understood, the presence of GABAA (1) and GABAB receptor sites (3) in the renal cortex suggests that via these receptor populations, GABA might modulate the tubular transport of chloride and/or calcium/potassium ions, respectively. Further studies are needed to clarify such intrinsic renal effects of GABA and related agonists.

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Literature Cited


Figure 5. Inner stripe of outer medulla, showing GABA-immunoreactive (white arrow) and non-reactive (black arrow) epithelial cells of the upper part of a long descending thin limb of Henle's loop. Bar = 1 μm

Figure 6. Epithelial cells of the distal tubule. The GABA-LI is seen in the infolded cytoplasm (white arrow), whereas mitochondria are not labeled. The neighboring cells are free of reaction product (black arrows). Bar = 1 μm

Figure 7. In collecting ducts the principal cell exhibits GABA immunostaining (white arrow), whereas the intracalated cell (black arrow) is free of reaction product. Bar = 1 μm

Figure 8. The endothelium of the peritubular capillary and three interstitial cell processes are positive for GABA-LI. Bar = 1 μm
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