Acetylcholinesterase-Containing Nerve Fibers in the Canine Kidney

By Olivia C. McKenna, Ph.D., and Evangelos T. Angelakos, M.D., Ph.D.

ABSTRACT

The distribution of acetylcholinesterase in the canine kidney was studied by the histochemical method of Karnovsky and Roots and compared to the distribution of adrenergic fibers studied with the fluorescence histochemical method of Falck and Hillarp. Specific inhibitors of nonspecific cholinesterase and acetylcholinesterase were used to differentiate between the two enzymes. In the hilus, acetylcholinesterase-containing nerve fibers were found in large bundles lying adjacent to the media of the hilar arteries. In the rest of the kidney, these fibers traveled with the interlobar, arcuate, and interlobular arteries, the afferent arterioles, and the vasa recta in the outer medulla. The outer medullary nerves appeared to originate in the arcuate connective tissue sheath. Acetylcholinesterase-containing nerve fibers were not seen in association with any other structures. The distribution was similar to that of adrenergic fibers although the density of the acetylcholinesterase-containing fibers was less. Two weeks after unilateral renal denervation, no adrenergic nerve fibers could be detected histochemically in the denervated kidney, whereas acetylcholinesterase-containing nerve fibers were found in five out of seven kidneys. Acetylcholinesterase-staining ganglion cells found in the hilus of the unsuccessfully denervated kidneys appeared to be the source of the acetylcholinesterase-containing nerves. On the basis of these observations, it may be concluded that the kidney is innervated by acetylcholinesterase-containing nerves which are independent of the adrenergic nerve supply.

ADDITIONAL KEY WORDS
cholinergic innervation histochemistry interlobar artery arcuate artery interlobular artery afferent arteriole vasa recta renal denervation

The adrenergic innervation of the canine kidney was studied recently in this laboratory using histochemical techniques (1). Adrenergic nerve fibers were found in association with the intrarenal arteries, afferent arterioles, and vasa recta. The possibility that the kidney may also be supplied by cholinergic nerves has been suggested by reports indicating that fibers from the vagus and pelvic nerves reach this organ (2-4). If cholinergic nerves are present in the kidney, they may be expected to have a high concentration of acetylcholinesterase as is true for cholinergic fibers found in other organs (5, 6). Nerves which stain for cholinesterase have been reported in the kidneys of rat, rabbit, cat, and human (7-9). However, the methods used were not always specific for acetylcholinesterase, and the relation between these fibers and the adrenergic innervation has not been studied. In an effort to compare the distribution of adrenergic nerves and acetylcholinesterase-containing nerves, we undertook an extensive histochemical study of the canine kidney. The thiocholine histochemical method of Karnovsky and Roots (10) with a modification...
Nerve fibers associated with the intrarenal arteries of the dog kidney as they travel from the hilus into the cortex. Acetylcholinesterase activity was demonstrated with the histochemical method of Karnovsky and Roots and an inhibitor of nonspecific cholinesterase. Catecholamine-containing nerve fibers were demonstrated with the fluorescence histochemical method of Falck and Hillarp. Magnifications: 160X.

A: Renal hilus. Acetylcholinesterase present in nerve fibers outside media of hilar artery (arrow) and in large nerve bundles (N). Counterstained with hematoxylin.

B: Interlobar artery after branching from the hilar artery. Catecholamine-containing nerves (arrow) appear as intensely fluorescent dots outside the media. Internal elastic membrane (iem) and connective tissue (ct)
ACETYLCHOLINESTERASE IN THE KIDNEY

Methods

Dogs were killed by an injection of sodium pentobarbital, 60 mg/kg, iv, and both kidneys were immediately removed. For the acetylcholinesterase studies pieces of the cortex, medulla, and hilus were frozen on the Freon plate in a Cryostat kept at −25°C. Frozen sections 10- to 30-μ thick were dried at room temperature for 5 to 15 minutes. Subsequently the slides were rinsed in distilled water and placed in the incubation medium described by Karnovsky and Roots (10) for 1 to 6 hours at 37°C. The final concentrations of the inhibitors used in the incubation medium were 8 x 10^-5 M tetraisopropylpyrophosphate (iso-OMPA), 10^-5 M diisopropyl fluorophosphate (DIFP), 10^-5 M 1,5-bis-(4-allyl dimethylammonium phenyl) pentan-3-one dibromide (284G51 Wellcome), and 10^-5 M eserine. Following incubation, the slides were either dehydrated and mounted directly or counterstained with hematoxylin (11).

To study the adrenergic innervation, adjacent sections were freeze-dried, exposed to formaldehyde gas, and studied in the fluorescence microscope. The remainder of each kidney was dissected into subcapsular cortex, mid-cortex, juxtamedullary cortex, outer medulla, and inner medulla. The noradrenaline and epinephrine levels in the five areas were analyzed fluorimetrically according to the method of von Euler and Lishajko (14). Details of these histochemical and chemical procedures were described in a recent publication (1).

Chronic denervation studies were performed on seven dogs. After sodium pentobarbital anesthesia, a left flank incision was made and the left kidney exposed. The nerves traveling with the renal vessels and the adventitia of the renal artery were stripped beginning at the hilus and extending toward the aorta. The nerves which were removed were fixed in formalin, embedded in paraffin, sectioned, and stained with Masson’s trichrome stain. In two dogs the nerves traveling with the ureter were also removed. The right kidney served as a control. After 2 weeks the animals were killed and both kidneys removed. Histochemical studies of the acetylcholinesterase activity and adrenergic innervation were performed as described above. Sections from right and left kidneys were incubated in the same medium to avoid variations that might be caused by slight differences in media.

Results

The specificity of the cholinesterase method of Karnovsky and Roots (10) was controlled by using several inhibitors. When no inhibitors were used, a positive reaction was found in neural structures as well as in the membranes of glomeruli, vasa recta, and tubule cells. No reaction product was found in any structure of the kidney when eserine, an inhibitor of both acetylcholinesterase and nonspecific cholinesterase (15), was included in the incubation medium. The results indicated that both acetylcholinesterase and nonspecific cholinesterase were demonstrated with this method. To differentiate between the two enzymes, specific inhibitors of each were tested. The use of 1,5-bis-(4-allyl dimethylammonium phenyl) pentan-3-one dibromide (284G51 Wellcome) as an inhibitor of acetylcholinesterase has been recommended (15). When acetylcholinesterase was inhibited with 284G51, nonspecific cholinesterase was found only in glomeruli, vasa recta, and tubule cells. In an effort to find a selective inhibitor for nonspecific cholinesterase, alternate sections were incubated with either iso-OMPA or DIFP. When DIFP was used, a weak reaction occurred in the glomeruli, vasa recta, and tubule cells. No reaction product was found in any structure of the kidney when eserine, an inhibitor of both acetylcholinesterase and nonspecific cholinesterase (15), was included in the incubation medium. The results indicated that both acetylcholinesterase and nonspecific cholinesterase were demonstrated with this method. To differentiate between the two enzymes, specific inhibitors of each were tested. The use of 1,5-bis-(4-allyl dimethylammonium phenyl) pentan-3-one dibromide (284G51 Wellcome) as an inhibitor of acetylcholinesterase has been recommended (15). When acetylcholinesterase was inhibited with 284G51, nonspecific cholinesterase was found only in glomeruli, vasa recta, and tubule cells. In an effort to find a selective inhibitor for nonspecific cholinesterase, alternate sections were incubated with either iso-OMPA or DIFP. When DIFP was used, a weak reaction occurred in the glomeruli, vasa recta, and tubule cells.

tubule cells and a strong reaction in the nerves, indicating that nonspecific cholinesterase had not been totally inhibited. In contrast, the reaction product was formed only in the nerves when iso-OMPA was used in the incubation medium. It was concluded that in the dog kidney iso-OMPA is a more effective inhibitor of nonspecific cholinesterase. Several authors have noted that the efficacy of DIFP as an inhibitor of nonspecific cholinesterase varies with the species and the tissue (15, 16).

On the basis of these studies, the distribution of acetylcholinesterase-containing nerve fibers was studied using iso-OMPA as an inhibitor of nonspecific cholinesterase. In the hilus, nerve fibers with acetylcholinesterase activity were found in large nerve bundles as well as immediately outside the muscularis and scattered throughout the adventitia (Fig. 1, A). Beyond the hilus the nerves traveled...
with the arterial supply as it subdivided. They
followed the interlobar arteries between the
pyramids to the arcuate arteries and to the
interlobular arteries of the cortex (Fig. 1, C-
E). Acetylcholinesterase-containing nerve fi-
bers were also found in association with the
afferent arterioles as they branched from the
interlobular artery (Fig. 2, A). No fibers were
seen entering the glomeruli or in association
with the afferent arterioles, veins, or tubules.

The nerve fibers associated with the intra-
renal arteries were often observed in bundles
which branched and formed a plexus around
the vessels (Fig. 1, E). In cross-section these
fibers were found between the media and
adventitia, never penetrating the smooth
muscle layers. In addition to the nerve fibers
which were closely applied to the arterial
smooth muscle, nerves were found scattered
in the connective tissue which surrounds the
hilar, interlobar, and arcuate vessels.

On a few occasions acetylcholinesterase-
containing nerves were observed accompan-
ying the vasa recta of the outer medulla.
Serial sections were used to determine the
source of these fibers. The outermedullary
nerves appeared to originate from the acetyl-
cholinesterase-containing fibers that were
scattered in the connective tissue sheath of
the arcuate vessels. They emerged from the
sheath in bundles and joined the vasa recta
of the outer medulla (Fig. 2, B).

The acetylcholinesterase-containing nerve
fibers were not found in association with all
arterial vessels. Although no quantitative meas-
urements were made, their frequency was
highest at the level of the hilar arteries and
decreased progressively at the interlobar,
arculate, and interlobular arteries. Such fibers
were found infrequently with afferent arteri-
oles and rarely with the vasa recta.

The effect of renal denervation on both
norepinephrine-containing and acetylcholines-
terase-containing nerve fibers were studied in
seven dogs. The animals were killed 2 weeks
after the renal nerves had been removed from
the left kidney. In the right control kidneys,
catecholamine-containing nerve fibers were
found accompanying the intrarenal arteries,
afferent arterioles, and vasa recta. The con-
centrations of norepinephrine in the five areas
of the right kidney are shown in Table 1. The
distribution of catecholamine-containing fi-
bers and the concentrations of norepinephrine
were similar to those reported in a previous
study of the adrenergic innervation of the
canine kidney (1) (Fig. 1, B and F). No
catecholamine-containing nerve fibers were
found histochemically in any area of the de-
nervated (left) kidneys. Furthermore, the
norepinephrine levels of all five areas fell to
less than 0.02 µg/g.

When the innervated (right) kidneys were
studied histochemically for the presence of
acetylcholinesterase-containing nerve fibers,
the distribution and density of these fibers
were similar to those in normal kidneys from
control animals. In two of the seven de-
nervated (left) kidneys, no acetylcholinester-
ase-containing nerve fibers could be found in
any region of the kidney. In the remaining
five denervated (left) kidneys, acetyl-
cholinesterase-containing fibers were seen in
association with the same structures as in the
control kidneys; however, there was a de-
crease in the number of these fibers. No
catecholamine-containing fibers could be
demonstrated in any of these kidneys, and no
norepinephrine could be found by chemical
analysis. Acetylcholinesterase-staining nerves
were also found in the two kidneys from
which the nerves of the ureter as well as those
of the renal vessels had been stripped.

When sections from the hilus of the partial-
ly denervated kidneys were examined for

### Table 1

<table>
<thead>
<tr>
<th>Area</th>
<th>Norepinephrine (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subcapsular cortex</td>
<td>0.24 ± 0.08</td>
</tr>
<tr>
<td>Midcortex</td>
<td>0.37 ± 0.13</td>
</tr>
<tr>
<td>Juxtamedullary cortex</td>
<td>0.61 ± 0.18</td>
</tr>
<tr>
<td>Outer medulla</td>
<td>0.41 ± 0.21</td>
</tr>
<tr>
<td>Inner medulla</td>
<td>0.03 ± 0.02</td>
</tr>
</tbody>
</table>

Values given are the means ± sd from seven kid-
neys.

Circulation Research, Vol. XXIII, November 1968
acetylcholinesterase activity, heavily stained nerve cells were found in the large nerve bundles present in this area (Fig. 2, C and D). The cells were always scattered along the nerve bundles and never aggregated into a ganglion. After adjacent sections had been treated histochemically for norepinephrine, no fluorescence characteristic of norepinephrine was found in these cells. The renal nerves removed at the time of denervation also contained acetylcholinesterase-containing nerve cells. When these nerves were stained with Masson's trichrome stain, small ganglia were found along their course.

Discussion

It is clear from the results that in the canine kidney nerve fibers which contain acetylcholinesterase are found in association with the intrarenal arteries, afferent arterioles, and vasa recta. These same structures were found to be innervated by adrenergic fibers in a previous study (1). The similar distribution of norepinephrine-containing and acetylcholinesterase-containing fibers may be taken to suggest that the adrenergic nerves contain acetylcholinesterase as proposed by Koelle (17) and that the kidney may not be innervated by cholinergic nerve fibers. The density of the adrenergic fibers is, however, much greater than that of acetylcholinesterase-containing fibers. This difference in density might be attributed to a less reliable method of demonstrating acetylcholinesterase or to a smaller number of nerves containing this enzyme.

After renal denervation all catecholamine-containing nerve fibers disappeared, and the norepinephrine concentrations in all areas fell to negligible levels indicating that total denervation of the adrenergic nerve supply was achieved. In five of these seven kidneys, acetylcholinesterase-containing nerves were still present although in smaller numbers. Other studies have indicated that 2 weeks after section of cholinergic nerves all acetylcholinesterase activity should have disappeared and the entire nerve degenerated (17-21). Therefore, the persistence of acetylcholinesterase-containing fibers in the kidney 2 weeks after denervation indicates that some of these nerves were derived from nerve cells peripheral to the site of denervation. Since stripping the nerves from the ureter did not affect the degree of denervation, it can be concluded that postganglionic acetylcholinesterase-containing nerves do not travel to the kidney via the ureter. The nerve cells found within the hilus of the partially denervated kidneys appeared to be the source of the remaining acetylcholinesterase-containing nerves. The nerves originating from these cells could not be adrenergic since complete denervation of the adrenergic nerve supply had been produced. On the basis of these studies it may be concluded that the kidney receives two types of nerve fibers—one containing norepinephrine and another containing acetylcholinesterase.

Successful denervation of the acetylcholinesterase-containing nerves in two of the seven kidneys can be attributed to a variation in the location of the nerve cells. Apparently all the cells contributing acetylcholinesterase-staining nerves to these two kidneys were outside the kidney and therefore susceptible to denervation. The occurrence of nerve cells along the renal nerves outside the kidney and within the hilus has also been noted in the human (3) and in the cat and dog (22). In general, the present results suggest that the acetylcholinesterase-containing fibers found in the canine kidney are cholinergic. Since these nerves are found only in association with blood vessels and since low concentrations of acetylcholine are known to produce renal vasodilatation (23-26), it would appear that excitation of these fibers would produce a decrease in vascular resistance.

It has been generally believed that the kidney does not receive cholinergic innervation since stimulation of the renal nerves under a variety of conditions has failed to produce renal vasodilatation (27). However, physiologic evidence for vasodilator innervation is beginning to accumulate. Studies using the sensitive Krypton 85 method for the detection of changes in renal blood flow have suggested...
that cholinergic vasodilator fibers function to maintain blood flow during reduction of renal arterial pressure (28). In addition, there is now evidence for a renal vasodilator reflex mediated by nerves which enter the spinal cord through the upper lumbar dorsal roots (29). It is evident that further studies are needed to elucidate the functional role of the acetylcholinesterase-containing nerves in the canine kidney.

Acknowledgment
The authors are indebted to Burroughs Wellcome & Co. (USA), Inc., for the gift of 1:5-bis-(4-allyl dimethylammonium phenyl) pentan-3-one dibromide (384C25).

References
Acetylcholinesterase-Containing Nerve Fibers in the Canine Kidney
OLIVIA C. MCKENNA and EVANGELOS T. ANGELAKOS

Circ Res. 1968;23:645-651
doi: 10.1161/01.RES.23.5.645

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1968 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/23/5/645

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at: http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at: http://circres.ahajournals.org//subscriptions/