Do distinct populations of dorsal root ganglion neurons account for the sensory peptidergic innervation of the kidney?

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Peptidergic afferent renal nerves (PARN) have been linked to kidney damage in hypertension and nephritis. Neither the receptors nor the signals controlling local release of neuropeptides [calcitonin gene-related peptide (CGRP) and substance P (SP)] and signal transmission to the brain are well-understood. We tested the hypothesis that PARN, compared with nonrenal afferents (Non-RN), are more sensitive to acidic stimulation via transient receptor potential vanilloid type 1 (TRPV1) channels and exhibit a distinctive firing pattern. PARN were distinguished from Non-RN by fluorescent labeling (DiI) and studied by in vitro patch-clamp techniques in dorsal root ganglion neurons (DRG; T11-L2). Acid-induced currents or firing due to current injection or acidic superfusion were studied in 252 neurons, harvested from 12 Sprague-Dawley rats. PARN showed higher acid-induced currents than Non-RN (transient: 15.9 ± 5.1 vs. 0.4 ± 0.2 pA/pF at pH 6; sustained: 20.0 ± 4.5 vs. 6.2 ± 1.2 pA/pF at pH 5; *P < 0.05). The TRPV1 antagonist capsazepine inhibited sustained, aminolide-transient currents. Forty-eight percent of PARN were classified as tonic neurons (TN = sustained firing during current injection), and 52% were phasic (PN = transient firing). Non-RN were rarely tonic (15%), but more frequently phasic (85%), than PARN (P < 0.001). TN were more frequently acid-sensitive than PN (50–70 vs. 2–20%, P < 0.01). Furthermore, renal PN were more frequently acid-sensitive than nonrenal PN (20 vs. 2%, P < 0.01). Confocal microscopy revealed innervation of renal vessels, tubules, and glomeruli by CGRP- and partly SP-positive fibers coexpressing TRPV1. Our data show that PARN are represented by a very distinct population of small-to-medium sized DRG neurons exhibiting more frequent tonic firing and TRPV1-mediated acid sensitivity. These very distinct DRG neurons might play a pivotal role in renal physiology and disease.

Classification of neurons; capacitance; TRPV1 channels; ASIC; renal afferent nerve; rat

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Nerves containing neuropeptides such as calcitonin gene-related peptide (CGRP) and substance P (SP) are important components of the sensory nervous system (13, 18). Although these afferent nerves until recently have been thought to sense stimuli in the periphery and transmit the information to the central nervous system, they also have an “efferent” local vasodilator function (10, 37). Acute administration of a CGRP receptor antagonist increases blood pressure in various models of hypertension, which indicates that this potent vasodilator plays a counterregulatory role to attenuate hypertension (12). Furthermore, CGRP was seen as nephroprotective in hypertensive kidney damage in other publications (3, 31). The release of neuronal peptides like CGRP is putatively dependent on the stimulation of transient receptor potential vanilloid type 1 (TRPV1) channels (30). But also in other organs, e.g., the liver, peptidergic afferent nerve fibers were able to release peptides to influence inflammatory and sclerotic processes (33). Hence, it is possible that CGRP release may be under the control of TRPV1 receptors also with respect to peptidergic afferents in the kidney, so that CGRP released from afferent renal nerve fibers can exert its nephroprotective potential due to its vasodilatory properties during inflammatory kidney disease, e.g., in hypertension (3, 31).

Concerning these important studies in animal models of hypertension pointing to considerable role of afferent sensory peptidergic nerve fibers and the TRPV1 channels, we are still lacking information about the afferent fibers and respective neurons involved, their specific characteristics, and in how far organ specificities with respect to sensitivity, e.g., in the kidney occur. It is widely accepted that tissue injury, inflammation, and ischemia are accompanied by local tissue acidosis (16), and TRPV1 (29) as well as members of the acid-sensing ion channels (ASIC) family (8, 42) have been described as putative transducers of peptidergic afferent nerves to interact with altered proton concentration. Are peptidergic renal nerves sensible or perhaps very sensible to stimulation by an increased proton concentration possibly via TRPV1 receptors or ASIC (30)? Such a finding could suggest mechanisms to be studied further to putatively explain neurogenic CGRP release being so effective in ameliorating hypertensive kidney damage (3, 31).

One major problem in this respect lies in the fact that afferent sensory nerve fibers, mainly C-fibers, are extremely difficult to record (4). Furthermore, recording of C-fibers does not allow to investigate distinct receptor mechanisms, since we are experimentally too much restricted to the thin fibers and not able to investigate the respective neurons or receptive endings (9). We recently presented results that showed in how far cultured sensory neurons from the dorsal root ganglia (DRG) with axonal projections from hindlimbs and kidneys allowed for the investigation of changes in mechanosensitivity of these very neurons in various models of secondary hypertension (19). Hence, we tested the hypothesis that distinct, clearly characterized neurons of the DRG with renal peptidergic afferents are more sensitive to the stimulation by protons possibly via TRPV1 receptors than neurons with nonrenal afferents from

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the very same DRG, that according to our previous work mainly obtain afferent input from the hindlimb vasculature (19). We used different proton concentrations in the culture medium as stimulus for TRPV1 since they are easily applied in cultured neurons and relevant with respect to inflammatory processes (19, 41). We measured inward currents using a standard patch-clamp equipment for electrophysiological investigations in the whole cell mode while voltage was clamped (20). The investigated neurons were grouped according to their capacitance. These groups corresponded quite well to the classes of morphologically distinguishable dorsal root ganglion neurons if sorted by size as described by others (15). To further substantiate the putative existence of a distinct population of renal afferent DRG neurons that are exceedingly sensitive to acidic stimulation, in a separate set of experiments the electrical and acidic excitability was investigated to classify the DRG neurons in terms of firing patterns as recently described by others (26, 27). In the latter experiments, current was clamped. Furthermore, we investigated kidney slices via confocal microscopy to demonstrate that all major parts of the kidney (glomeruli, tubuli, vessels) were innervated by peptidergic afferent nerve fibers (33).

METHODS

Male Sprague-Dawley rats (Ivanovas, Kisslegg, Germany) weighing 250–300 g were maintained in cages at 24 ± 2°C. They were fed a standard rat diet (no. C-1000, Altromin, Lage, Germany) containing 0.2% sodium by weight and were allowed free access to tap water. All procedures performed in animals were done in accordance with the guidelines of the American Physiological Society and approved by the local government agency (Regierung von Mittelfranken, Ansbach, Germany).

Labeling of dorsal root ganglion neurons with renal afferents. To identify neurons with putative renal afferents, these cells were labeled using the dicarbocyanine dye DiI (1,1'-dioleoyl-3,3,3'-tetramethyldiocarbocyanine methansulfonate, 39-DiI, DiI, 50 mg/ml in DMSO; Molecular Probes, Eugene, OR) by subcapsular application of DiI (5 μl; 10 mg/ml) in both kidneys 1 wk before harvesting neurons from DRG T11-L2 (5, 20). Care was taken to prevent back leak or spreading of DiI to surrounding tissue. To this end, rats were anesthetized with intraperitoneal bolus injection of methohexital (500 μl; 25 mg/ml); additional doses were given when appropriate. We allowed 1 wk for DiI to be transported retrogradely to the neuronal cell bodies residing in the DRG. The subcapsular renal DiI application could be brought about with minor surgery. Only small flank incisions through skin and muscles were necessary to expose renal poles. Analgesic drugs were not necessary and the animals recovered readily.

Neuronal cell culture. Respective rats were anesthetized with methohexital as described above, the animals were decapitated, and DRG from T11-L2 was dissected. Primary cultures of neurons from the DRG were obtained by mechanical and enzymatic dissociation by adapting protocols previously described by others (6). The ganglia were incubated with collagenase (1 mg/ml), trypsin (1 mg/ml), and DNase (0.1 mg/ml) and resuspended in modified L-15 medium for 1 h at 37°C that contained 5% rat serum and 2% chick embryo extract as well as necessary inorganic salts, amino acids, and vitamins (Sigma-Aldrich, Munich, Germany). Enzymatic activity was terminated by the addition of soybean trypsin inhibitor (2 mg/ml), bovine serum albumin (1 mg/ml), and CaCl₂ (3 mM) in modified L-15 medium, and the ganglia were triturated using sterile siliconized Pasteur pipettes to dissociate individual cells. After centrifugation, the cells were again resuspended in a modified L-15 medium with 5% rat serum and 2% chick embryo extract and plated on poly-L-lysine-coated glass coverslips. 5-Fluorodeoxy-2-uridine (80 μM/l) was added to suppress the proliferation of nonneuronal cells especially fibroblasts. The cells were cultured on coverslips for 1 day for electrophysiological experiments again in a modified t-15 solution (20). To demonstrate that labeled cells were neurons, all cells used for experimental procedures were tested for fast sodium currents during repolarization that are characteristic for neuronal cells. A laser beam (532 nm) was mounted to the patch-clamp recording set-up to allow for the detection of DiI-stained DRG cells during the experiments using respective optical filters. From previous experiments, we knew that the nonrenal neurons in the DRG dissected have to the greatest extent axonal input from the hindlimbs of the experimental animals (19).

Patch clamp. Patch recordings (19) were obtained from respective neurons using a recording solution containing (in mM) 104 KCl, 16 KOH, 1 magnesium ATP, and 10 HEPES. Electrode resistance was 3–6 MΩ, seal resistance was 2–10 GΩ, and series resistance was >100 MΩ. Whole cell voltage-clamp recordings were obtained with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Data were sampled at 5 kHz for voltage-clamp protocols and 20 kHz for current-clamp protocols, filtered at 2 kHz, and stored on a computer hard disk using a commercially available software package (pClamp 8.2, Axon Instruments). We only included neurons in the analysis if their resting membrane potentials were below ~40 mV. All experiments described in our study were done in whole cell mode. Only cells that stained brightly for DiI with laser excitation were accepted as neurons with renal axons used for the experiments after harvest and culturing. The experimental protocol was also performed in neurons with nonrenal afferents likewise located in DRG of vertebrae T11-L2 to use the results from a neuron population with afferents of a different anatomical site as comparison, that according to our previous work represented afferent innervation first and foremost from the animals’ hindlimb as already mentioned above (19). All recordings were done at room temperature, i.e., 24°C, as described by others (26, 27).

Voltage-clamp protocols. Acid-sensitive currents were induced by superfusing the respective neuron under investigation for 10 s with solutions of either pH 6 or pH 5 once every minute, respectively, using a multibarrel perfusion pipette that was connected to computer-controlled magnetic valve system controlling the flow of gravity-fed lines that were connected to reservoirs containing the respective solutions. Every cell was exposed to both solutions that were administered to the bath in random order; pH was adjusted to 7.4 with NaOH and to 6 and 5 with HCl. 2-N-morpholinoethanesulfonic acid (MES) was used instead of HEPES in the acidified solutions. The observed current/time relationship during acidic stimulation of the neurons showed a characteristic pattern consisting of a transient and a sustained component (see Fig. 1A). The TRPV1 channel inhibitor capsaicin (32) and amiloride (21), an unselective inhibitor of ASIC, were used to clearly distinguish and characterize TRPV1 and ASIC-dependent currents, respectively. Currents were adjusted to cell capacitance to express current densities. Whole cell capacitance as an indicator for cell surface was related to a classification of the cells by their size as assessed through the microscope using an ocular-mounted micrometer caliper.

Current-clamp protocols. To allow for a classification of DRG neurons with renal vs. nonrenal afferent projections (i.e., DiI-positive or DiI-negative, respectively) in terms of firing patterns, we used an adaptation of a current-clamp approach recently described by others (26, 27). Action potentials in response to current injection were recorded by use of an Axopatch 200B amplifier (Axon Instruments). The pClamp 8.2 software (Axon Instruments) was used to control current-pulse generation, to record membrane potentials, as well as for off-line data analysis. Action potentials were induced by rectangular current-pulse injections as follows: a 5 ms pulse, followed by a 600-ms pulse with an interpulse delay of 100 ms was delivered in two consecutive trains of stepwise increased intensity; 40–400 and 400–4,000 pA, in 10 consecutive steps, with each step lasting 5.16 s. This
channels in renal dorsal root ganglia (DRG) neurons (the expression of transient receptor potential vanilloid type 1 (TRPV1) blocked with capsazepine in a dose-dependent manner (even during very high levels of current injection.

Beginning of the period of depolarizing current injection are observed, activation means that only a single or a few action potentials at the period of depolarizing current injection. In contrast, phasic activation means generation of repetitive action potentials that are sustained at a rather constant rate throughout “phasic” (26, 27): tonic activation means that acid-induced transient as well as sustained currents could be blocked with amiloride in a dose-dependent manner, which points to the expression of acid-sensing ion channels (ASIC) in renal DRG neurons (n = 7).

Protocol allowed to categorize each DRG neuron as “tonic” or “phasic” (26, 27): tonic activation means generation of repetitive action potentials that are sustained at a rather constant rate throughout the period of depolarizing current injection. In contrast, phasic activation means that only a single or a few action potentials at the beginning of the period of depolarizing current injection are observed, even during very high levels of current injection.

After a recovery time of at least 5 min, when the resting membrane potential returned to its individual baseline value, each cell was superfused with solutions of either pH 6 or pH 5 once in a minute, for 10 s in randomized order, over a period of 8 min. When action potentials were generated in response to acidic stimulation, either sporadic or in regular trains, the respective DRG neuron was categorized as acid sensitive; neurons that remained silent were categorized as acid resistant.

Immunohistochemistry. For detection of sympathetic and sensory nerve fibers, immunocytochemistry for tyrosine hydroxylase (TH) and SP or CGRP, respectively, as well as TRPV1 receptors was utilized (2, 25, 40). Twelve-micrometer-thick sections from three paraformaldehyde perfusion-fixed rat kidneys (10 sections from each kidney, spaced 60 µm apart) were incubated for single or double immunofluorescence with primary antibodies (sheep anti-TH, Novus Biologicals, Littleton, CO, 1:2,000; rabbit anti-SP, Peninsula, San Carlos, CA, 1:750; rabbit anti-CGRP, Peninsula, 1:1,000; goat anti-CGRP, Biotrend, Köln, Germany, 1:250; rabbit anti-TRPV1, Calbiochem/ Merck, Darmstadt, Germany, 1:400) dissolved in Tris-buffered saline (TBS; pH 7.33) overnight at room temperature. After three rinses in TBS, appropriate fluorochrome-tagged secondary antibodies (donkey anti-goat and anti-rabbit IgGs tagged with either Alexa 488 or Alexa 555, 1:1,000 in TBS, all from Molecular Probes) were applied for 1 h at room temperature. Controls included omission of primary antibodies, replacement of primary antibodies with the respective normal serum, and incubations with primary antibodies preadsorbed with the respective antigen. Sections were investigated using a Bio-Rad MRC 1000 confocal system attached to a Nikon Diaphot 300 inverted microscope. The blue 488-nm and yellow 568-nm lines of a krypton-argon laser were used for excitation of Alexa 488 and Alexa 555, respectively. The blue 488-nm laser line was also used for eliciting green autofluorescence of various tissue elements in the kidney and thus used as counterstain. Merged two-channel confocal images were adjusted for contrast and brightness using Adobe Photoshop.

Data analyses. The data were statistically analyzed with ANOVA and Newman-Keuls post hoc test, or t-test (where appropriate) using a CSS statistical software package (StatSoft, Tulsa, OK). Only a priori fixed comparisons were tested. Statistical significance was defined as P < 0.05. Data are given as means ± SE (28). Furthermore, z-test was used to test for significant differences in the frequency distribution of characteristics of the DRG neurons (e.g., tonic, phasic, acid sensitive, acid resistant).

RESULTS

Labeled cells in situ and in culture. In slices of harvested DRG, those cells that were positively stained with DiI could be viewed under epifluorescence with modulation contrast optics as red-orange cells. A clear difference in the pattern of distribution between neurons with renal or nonrenal afferents could not be observed, although the neurons with renal afferents, i.e., DiI positive, appeared to be found more often closer to the ventral portion of the ganglia. After 1 day in culture, dorsal root ganglion cells could be distinguished in most cases from fibroblast and other cells by their larger soma. A staining with DiI could be observed in 40% of cultured neurons, suggesting afferent axonal input from the kidneys. A fraction of these cells (between 15–20% of all cultured cells) was brightly labeled with DiI. These brightly labeled cells were defined as renal afferent DRG neurons and were compared with clearly unlabeled neurons which received afferent input from nonrenal sites. No significant differences in size could be detected between labeled and unlabeled cells.

Voltage-clamp protocols. Renal (i.e., DiI labeled) as well as nonrenal (i.e., unlabeled) DRG neurons mostly showed the complex pattern of acid-induced transient as well as sustained currents at pH 6 and pH 5 (Fig. 1A).

The sustained component of the acid-induced inward current could be blocked by capsazepine in a dose-dependent manner at pH 6 and pH 5 which points to the existence of TRPV1 receptor channels in these cells (Fig. 1B). No effects of amiloride could be observed on the sustained currents.

Additionally, observed transient currents could be blocked by amiloride at pH 5 and pH 6 indicating the existence of ASIC-type channels on the DRG neurons under investigation (Fig. 1C). The transient currents were not different at either pH (Fig. 1A). No effects of capsazepine could be observed on transient currents.
Neurons could be readily divided in three groups of capacitance (<80, 80–160, >160 pF) corresponding to small-, medium-, and large-sized renal and nonrenal neurons (<30, 30–45, >45 μm) as described by others (15).

In the sample of DRG neurons that underwent the voltage-clamp protocol (n = 72), the largest group of cells is represented in the medium-capacitance, medium-sized group (Fig. 2). The TRPV1 occurrence as assessed by the respective sustained current induced by proton stimulation, which could readily be blocked by the TRPV1 antagonist capsazepine, was significantly higher in renal than in nonrenal cells with respect to the medium- and large-capacitance neurons, but no difference occurred with respect to the small-capacitance ones (Fig. 2A). Likewise in medium- and large-capacitance renal neurons, ASIC seem to be significantly more expressed than in nonrenal neurons. In small-sized cells with respective capacitance, no ASIC currents could be observed regardless of the original site of afferent innervation, i.e., renal or nonrenal, respectively (Fig. 2B).

**Current-clamp protocols.** Since the voltage-clamp approach strongly indicated the existence of a functionally distinct population of DRG neurons that account for the renal afferent innervation, current-clamp experiments were done to further classify the T11-L2 DRG neurons. Based on DiI labeling, renal DRG neurons (n = 96) were compared with nonrenal DRG neurons (n = 84). Forty-eight percent of renal neurons could be classified as “tonic neurons” (TN = sustained AP generation during depolarizing current injection); 52% were classified as “phasic neurons” (PN = transient AP generation during depolarizing current injection). Nonrenal DRG neurons were far less tonic (15%), but more often phasic (85%, P < 0.001). Furthermore, acid sensitivity was found much more frequently in TN than in PN (50–70 vs. 2–20%, P < 0.01). Moreover, even renal PN were more frequently acid sensitive than nonrenal PN (20 vs. 2%, P < 0.01). These findings are illustrated in Fig. 3. Renal TN tended to be larger than nonrenal TN in terms of capacitance (92.9 ± 7.5 vs. 56.8 ± 7.2 pF, not significant); however, this difference failed statistical significance. In acid-sensitive neurons of any group, membrane depolarization due to the same acid load was much more pronounced (Δ35.7 ± 2.4 vs. Δ13.9 ± 1.2 mV, P < 0.0001). Thus, the current-clamp approach strongly substantiates the existence of a functionally distinct group of renal afferent DRG neurons of medium-to-large size. Baseline membrane parameters from the cells investigated in the current-clamp experiment (renal vs. nonrenal) are given in Table 1. All cells, phasic and tonic, exhibited phasic responses at low-current injection levels. This current level needed to induce a phasic response was the same in renal an nonrenal neurons; however, the membrane threshold potential for phasic response was significantly more negative in nonrenal cells, and the change in membrane potential was more pronounced in renal neurons. Furthermore, the shift in membrane potential due to acidic stimulation was more pronounced in renal cells (Table 1).

When the data obtained from current-clamp experiments were grouped by size as above (<80, 80–160, >160 pF), the frequency distribution of acid sensitivity was significantly higher in small- and medium-sized renal neurons (Fig. 4). The difference in membrane potential shift due to acidic stimulation was similarly distributed between capacitance groups (Fig. 5).

**Confocal microscopy.** Renal nerves containing bundles of sympathetic TH-positive and sensory primary afferent axons specifically stained for CGRP and SP entered the kidney along the arterial tree. As a rule, SP-positive axons were less frequent than CGRP-positive fibers. Typically, sympathetic axons (stained red in Fig. 6, left) and primary afferent CGRP-positive axons (stained green in Fig. 6, left) were coursing side by side within the same bundles sometimes resulting in yellow mixed color due to overlay. The wall of the renal pelvis was densely innervated by both sensory and sympathetic fibers (data not shown). Both types of nerve fibers ramified in the renal cortex, whereas the medulla was devoid of terminal axons. In every section investigated, all components of the cortex, i.e., glomeruli, tubules, and blood vessels, were contacted by these nerve fibers (Fig. 6). Almost all of the CGRP-positive primary afferent nerve fibers costained for TRPV1 receptor and vice versa (Fig. 7). These nerve fibers were seen both along arteries as well as in the interstitium between tubules where they ramified (Fig. 7, middle). Controls did not show any specific immunostaining. Furthermore, we could show recently that after renal denervation all these nervous structures described above were no longer detectable (35).
DISCUSSION

We could demonstrate for the first time that subgroups of sensory peptidergic neurons with renal afferents likely involved in renal function and inflammatory processes within the kidney are distinctively more sensitive to stimulation with protons compared with neurons with axons from other sites. Furthermore, this greater susceptibility of renal afferent neurons to acidic stimulation correlated with a higher rate of tonic firing response due to depolarizing current injection into respective neurons in culture. Vice versa, nonrenal neurons

Table 1. Renal and nonrenal parameters

<table>
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<th>Parameter</th>
<th>Units</th>
<th>Renal (n = 96)</th>
<th>Nonrenal (n = 84)</th>
<th>Statistics</th>
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<tr>
<td>Revers-Mpot</td>
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<td>−51.2±0.6</td>
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<td>Cm</td>
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<td>Rm</td>
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<tr>
<td>Rest-Mpot</td>
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<td>−52.0±0.79</td>
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<td>Phasic Thres-pot</td>
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<td>Tonic Thres-pot</td>
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<td>∆Mpot phasic</td>
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<td>Phasic current</td>
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<td>∆Mpot pH 5</td>
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<td>24.5±0.8</td>
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Numbers are given as means ± SE. Revers-Mpot, reversal potential; Cm, membrane capacitance; Rm, membrane resistance (Cm and Rm measured at −80-mV holding potential, −5-V rectangular test pulse, 5 Hz); Rest-Mpot, resting potential; Phasic Thres-pot, threshold potential for phasic response; Tonic Thres-pot, threshold potential for tonic response; ∆Mpot phasic/∆Mpot tonic, difference between resting potential and phasic or tonic threshold potential, respectively; Phasic current/tonic current, current injected for phasic or tonic threshold response, respectively; ∆Mpot pH 6/∆Mpot pH 5, membrane potential shift due to acidic stimulation (pH 5/pH 6); ns, not significant.

Fig. 4. Acid sensitivity obtained from current-clamp experiments in renal (black bars) and nonrenal neurons (white bars) divided in the 3 groups according to cell size, based on their membrane capacitance. Small (<80 pF)- and medium-sized (80–160 pF) renal cells were significantly more frequently sensitive to acidic stimulation in terms of action potential generation. *P < 0.01, renal vs. nonrenal.

Fig. 5. Change of membrane potential due in renal (gray/black) and nonrenal (white) neurons divided in the 3 cell size groups, based on their membrane capacitance, due to acidic stimulation (pH 6 and pH 5). Again, small (<80 pF)- and medium-sized (80–160 pF) renal cells were significantly more sensitive to acidic stimulation in terms of membrane potential change. *P < 0.01, renal vs. nonrenal.
exhibited far more frequently a phasic firing pattern while being far less frequently acid sensitive.

Acidic stimulation with protons stimulated predominantly neuronal TRPV1 receptors on respective neurons but also ASIC, distinguishable by the use of the respective channel blockers capsazepine and amiloride.

The idea to categorize peptidergic afferent sensory neurons in terms of proton susceptibility was based on the following considerations: a major and unique task of the kidney is to excrete an amount of acid equal to daily production to maintain systemic acid-base balance (24). Thus, the existence of some neuronal acid sensory system suggests itself. Furthermore, the importance of an acidic milieu in the kidney is likely further enhanced during inflammatory processes in nephritis and renal hypertensive disease, when respective afferent nerve fibers from the kidney might play an important role in the pathology of these diseases: generally, tissue injury, inflammation, or ischemia is accompanied by local tissue acidosis (16), and TRPV1 (29) and ASIC (8, 42) have been described as putative transducers on peptidergic afferent nerves. Furthermore, although being less specific proton stimulation does not show tachyphylaxis and desensitization as the specific TRPV1 ligand capsaicin.

The neurons investigated were not morphologically homogeneous, but could be classified, as described before (15, 36), according to cell size into three groups, that approximately correlated with neuronal capacities measured in our experiments. The mentioned studies on which we based this "size classification" could show direct correlation of neuronal soma size with peripheral nerve conduction velocity in L4 DRG: summarized, small- and medium-sized (mainly B type or small dark) neurons mostly give rise to unmyelinated C and thin myelinated Aβ-fibers, whereas large (mainly A type or large light) neurons give rise to Aα and Aβ fibers (15). There is, however, some overlap in cell size, mostly in Aδ fibers. In general, sensory neurons innervating inner organs are composed mainly of the peptidergic subpopulation of B-type primary afferent neurons (22, 35).
The group of neurons with medium size and capacity likely to be polymodal and hence responsible for mechano- and chemoception (15, 34) is thus probably linked mainly to C fibers. In this group, renal neurons were not only more sensitive to acidic stimulation in terms of action potential generation than neurons with projections from other, nonrenal sites but showed also higher membrane currents after acid-potentiators stimulations with protons, which was the case for TRPV1- as well as for ASIC-mediated currents, as shown by use of the respective channel blockers capsaizpine and amiloride. Higher membrane currents upon stimulation with protons might suggest a generally higher responsiveness of cells also with respect to other cellular processes than action potential generation. Such processes may also encompass the release of CGRP and SP from axonal nerve endings within the kidney already described often for these neurons.

However, this question must be addressed in further specific experiments for the kidney. Such studies will then also have to take into account the recent finding in DRG neurons (from segments L4-S3) that neurokinines, like SP (26, 27), are able to enhance the excitability of capsaicin-responsive (i.e., TRPV1 expressing) neurons via neurokinin receptor 2 (NK2) when cells were stimulated by current injection or capsaicin. It is likely that such a positive feedback system plays a significant role in the kidney in health and disease. It remains, however, to be determined whether such feedback mechanisms are detectable in proton stimulation.

The group of neurons with small size and low capacity is often seen as involved into noci- and thermoception (15). Renal cells in that group were also more sensitive to acidic stimulation with respect to action potential generation. Since these modalities are poorly defined for the parenchymal part of the kidney, the putative role of these afferent renal neurons is somewhat elusive. They may serve comparable regulatory purposes as renal medium-sized neurons, but the fact that small-sized neurons with renal axons were not unique when it comes to membrane currents as compared with small-sized neurons with nonrenal afferents suggests that these neurons cannot be easily compared with neuronal cells of medium size. Of note is that these cells did not exhibit ASIC-mediated currents.

The group of renal neurons with large size, high capacity, and putatively high conduction velocity is rather likely to innervate the renal capsule than subserving any role in renal cortex or medulla. Conceivable is also nociception from the renal pelvis, if we take into account what is known from neurons with axons exhibiting the modalities of Aα and Aβ fibers (36).

Our morphological studies revealed that afferent peptidergic nerve fibers were mostly in close vicinity to sympathetic efferent nerve fibers. It is known that biogenic amines required for neurotransmission are transported into respective storage vesicles utilizing an electrochemical gradient across the vesicular membrane established by proton pumping into the vesicle via a vacuolar ATPase (11). Hence, the vesicles having a membrane impermeable to protons must contain a considerable amount of protons to be able to accumulate singly positively charged amines into the relatively proton-impermeable acidic secretory vesicles at the expense of proton antiport through a transporter protein (23). This means that release of biogenic amines from the vesicles will increase locally the proton concentration that could stimulate TRPV1 and ASIC receptors on afferent nerve fibers in the close vicinity. The putative relevance of such a mechanism in health and disease needs further evaluation.

In a recent study, renal afferent innervation was linked to the amelioration of the consequences of oxidative stress (38). In rats with destruction of afferent sensory nerves due to early postnatal capsaizpine treatment, enhanced $O_2^-$ could be measured in the cortex and medulla of the kidney if these animals were on a high-salt diet. Increased superoxide levels in these animals were attributed to increased expression and activity of NAD(P)H oxidase, which was thought to contribute to decreased renal function and increased blood pressure. Since oxidative stress is connected with decreased vasodilatation and reduced tissue perfusion (7), altered regional proton concentration in the kidney under these circumstances could stimulate TRPV1 receptors to promote vasodilatory CGRP release.

It is known that protease-activated receptor 2 (PAR2) sensitizes TRPV1 receptors (1). PAR2 expression was observed in renal tissues like IgA nephropathy potentially aggravating the pathogenesis of interstitial fibrosis (14, 39). Furthermore, since TRPV1 receptors are eventually stimulated not only by protons or sensitized by PAR2 but affected by a large variety of substances and metabolic products potentially involved in inflammatory responses (17), it is possible that also a wide variety of different pathophysiological circumstances with inflammation and progressive fibrosis will stimulate these receptors. Since we are now able to classify and investigate specific afferent sensory peptidergic neurons with projections to important cardiovascular organs like the kidney and have enough evidence that afferent peptidergic nerve fibers play a role in the pathophysiology of inflammation, it is now important to clarify the specific mechanisms in target organ damage and inflammation that lead to altered expression and release of neuronal peptides like CGRP. Specifically, it will be challenging to figure out which of the various possibilities of TRPV1 receptor stimulation is of actual importance in this context to eventually develop respective therapeutic strategies beneficially influencing these neurogenic systems involved in hypertension.
RENAL SENSORY AFFERENTS

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