Expression of KCNA10, a Voltage-Gated K Channel, in Glomerular Endothelium and at the Apical Membrane of the Renal Proximal Tubule

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Abstract. Potassium (K) channels regulate cell membrane potential and modulate a number of important cellular functions. KCNA10 is a cyclic nucleotide-gated, voltage-activated K channel that is detected in kidney, heart, and aorta by Northern blot and postulated to participate in renal K metabolism and to regulate vascular tone. The aim of this study was to establish the cellular and subcellular localization of KCNA10 in kidney and vascular tissues. An anti-KCNA10 polyclonal antibody was generated, and immunocytochemical studies were performed on rat kidney. KCNA10 protein was easily detectable at the apical membrane of rat proximal tubular cells, and a weaker signal was also evident in the glomerulus. *In situ* hybridization experiments confirmed the immunocytochemical studies and revealed *KCNA10* expression in human proximal tubular cells, glomerular and vascular endothelial cells, and also in vascular smooth muscle cells. The data suggest that KCNA10 may facilitate proximal tubular sodium absorption by stabilizing cell membrane voltage. Furthermore, its presence in endothelial and vascular smooth muscle cells supports the notion that it also regulates vascular tone.

Potassium (K) channels are membrane proteins that participate in many cellular processes primarily by regulating membrane potential. KCNA10 is a voltage-gated K (Kv) channel gene related to the *Shaker* superfamily (1), and its most distinguishing feature relates to the presence of a putative cyclic nucleotide-binding (CNB) domain at the carboxy terminus. A few other K channels also contain CNB domains and may belong to new subclass of K channels with structural features common to both Kv channels and to cyclic nucleotide-gated cation channels (2–5). For instance, the HERG channel binds cAMP, regulates cardiac repolarization, and is mutated in some cases of long QT syndrome (6,7). Other potential physiologic roles for these proteins include arterial vasorelaxation (8), hormone secretion (9,10), and neuronal membrane excitability (11).

The α subunit of KCNA10 (αKCNA10) (1) mediates voltage-gated K currents that exhibit minimal steady-state inactivation. Channels are activated by depolarizations more positive than -66 mV. The channel displays an unusual inhibitor profile, because in addition to being blocked by classical K channel blockers, it is also sensitive to inhibitors of cyclic nucleotide gated cation channel such as verapamil and pimozide. Tail current analysis indicates that KCNA10 has a potassium-to-sodium selectivity ratio of at least 15:1. The single channel conductance is approximately 11 pS, and channel activity is inhibited by protein kinase C.

Numerous studies have shown that the properties observed for cloned α subunits very rarely match currents known to exist in native tissues (12). Most often it is because the α subunits interact with accessory proteins that modify their kinetic and pharmacologic properties. We recently identified an accessory subunit (KCNA4B) of KCNA10 by yeast-2-hybrid using the C terminus of the KCNA10 as bait. KCNA4B binds to the C terminus of KCNA10 and can be immunoprecipitated *in vitro* and *in vivo*. The biologic significance of this interaction is reflected in the fact that KCNA4B increases KCNA10 current expression by at least 2.8-fold and also alters its sensitivity to cAMP.

In previous studies, we documented *KCNA10* expression by Northern blotting and PCR in renal tubular cells and blood vessels vascular tissue (13,14). PCR of dissected nephron segments suggested widespread expression of KCNA10 because a signal was observed in all nephron segments tested, including glomeruli, proximal tubule, connecting tubule cortical collecting ducts, and renal vessels. There were three significant limitations of the PCR analysis. First, the primers used for analysis resided on the main exon of KCNA10, and we could not conclusively rule out genomic contamination. Second, we could not exclude the possibility that dissected tubule segments were contaminated by other tissues, including blood vessels. Lastly, PCR analysis could not ascertain if the protein was actually expressed and if expression was basolateral or apical. The task of ascribing a definitive functional role to KCNA10 will be greatly facilitated by accurate and precise knowledge of channel expression at the nephron segment, cellular, and membrane level; we therefore generated an anti-

Received June 20, 2002. Accepted August 10, 2002.
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1046-6673/1312-2831
Journal of the American Society of Nephrology

DOI: 10.1097/01.ASN.0000036866.37886.C5

KCNA10 polyclonal antibody and used it to localize KCNA10 in the kidney.

Materials and Methods

Antibody Preparation

Polyclonal sera were raised in rabbits by Biosynthesis Inc. (Lewisville, TX) against a peptide corresponding to amino acids 47 to 65 (SNWRVLISDNTNHETAFSK) of mouse KCNA10 (mKCNA10). The sera were purified before use by affinity chromatography using a column containing the mKCNA10 peptide.

In Vitro Translation

The coding regions of hKCNA10 (accession No. U96110) and hKv1.3 (accession No. XM084080) were amplified by PCR with a proofreading polymerase and sense primers containing a T7 polymerase site. The PCR products were used as template for cRNA synthesis using T7 polymerase (Ambion), and *in vitro* translation was carried out for each individual cRNA using rabbit reticulocyte lysates (Ambion).

Preparation of Renal Membrane Vesicles

Adult male Harlan Sprague-Dawley rats (Charles River) were sacrificed by injection of sodium pentobarbital (Butler Co., Columbus, OH), and microvillus membrane vesicles were prepared from renal cortex using the $MgCl_2$ precipitation method as described previously (15).

Western Blot Analyses

Protein samples were solubilized in SDS-PAGE sample buffer and separated by SDS-PAGE using 7.5% polyacrylamide gels. For immunoblotting, proteins were transferred to polyvinylidene difluoride (Millipore Immobilon-P) at 500 mA for approximately 10 h at 4°C with a TransphorTM transfer electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA). The blot was stained with Ponceau S in 0.5% trichloroacetic acid, and nonspecific binding was blocked by incubation in Blotto (5% nonfat dry milk in phosphate-buffered saline [PBS], pH 7.4) for 1 to 3 h. Blots were then incubated for 1 to 5 h with anti-KCNA10, the primary antibody, diluted in Blotto, at dilutions ranging from 1:200 to 1:1000. In some studies, (peptide competition), the primary antibody was preincubated with 100 ng of the antigenic peptide for 1 h before it was added to the blot. The blots were then washed in Blotto and incubated for 1 h with an appropriate horseradish peroxidase-conjugated secondary antibody diluted 1:2000 in Blotto. The blots were than thrice washed: once with Blotto, once in PBS (pH 7.4), and once in distilled water. Bound antibody was detected by ECLT chemiluminescence system (Amersham Pharmacia Biotech) according to the manufacturer's protocols.

Immunolocalization

For light microscopic studies, Epon sections (0.5 mm) are cut with a glass knife, stained with toluidine blue, and examined with a Zeiss Axiophot microscope. Fixed tissue was embedded in paraffin, and 0.5- to 2.0-mm-thick sections were cut using a glass knife mounted on the sectioning stage of a Reichert ultramicrotome. Sections were then mounted on glass coverslips, deparaffinized using xylene, and rehydrated in graded ethanols and PBS. Sections were microwaved in buffer containing 10 mM citrate in Tris-buffered saline (TBS) at 40% power for 20 min. After washing in TBS, the sections were further denatured using 1% SDS in TBS for 5 min. After washing in TBS, the sections were immunolabeled with the primary antibody at a dilution of 1:100 or with the preimmune serum (1:100 dilution). They were washed in blocking solution and then incubated for 1 h at room temperature with an FITC-labeled goat, anti-rabbit secondary antibody (Zymed Laboratories, South San Francisco, CA) at a dilution of 1:100. Finally, the slides were washed in PBS, preserved in crystal/mount (Biomeda, Foster City, CA), and examined within 4 h using a Zeiss fluorescence microscope. Representative pictures were recorded and stored digitally.

In Situ Hybridization

A 334-bp fragment of KCNA10 (1), −4 to +330, (GenBank accession No. U96110; start Codon counted as position 1) was amplified by PCR using the following primers: sense 5'-TAGAATGGATGTGTGTGGCTGGAA-3' and antisense 5'- GTCTCTGGGAACTGACTAAGGGTT-3'. That region is highly specific for KCNA10, and a GenBank search revealed no significant homology to other known genes. The amplified product was cloned into pPCR-ScriptAmp cloning vector (Stratagene), and the authenticity of the clone was confirmed by automatic DNA sequencing using ABI 310 autosequencer (Perkin Elmer). The clone was made linear by digestion with *Not*I, and a DIG-labeled antisense riboprobe was synthesized with T7 RNA polymerase using a DIG-labeling kit (Roche Biochemicals, Germany). The sense ribopobe was synthesized by digesting with *Eco*RI and transcribing with T3 RNA polymerase. An adult human kidney and cerebral artery were obtained from an autopsy case with written consent of family members under an approved protocol at the Chinese University of Hong Kong. Tissues were fixed overnight with 4% paraformaldehyde in PBS. We examined tissues from two deceased subjects whose bodies were immediately transferred to cold room and kept a 4°C. Autopsy was done within 5 to 7 h. There were no differences with regards to KCNA10 expression. The data presented in the Figures were obtained from the tissues of a 59-yr-old woman who died of cardiac tamponade caused by a dissecting aortic aneurysm. Her kidneys showed only mild, age-related nephrosclerosis, and she had no history of hypertension. The protocol that follows is a modification of that of Yew *et al.* (16). The tissues were dehydrated through graded ethanol, cleared with xylene, embedded in paraffin, and cut into 6-μm-thick sections. The sections were washed briefly with diethylpyrocarbonate-treated water followed by PBS for 10 min. They were then digested with proteinase K (10 μg/ml) at 37°C for 10 min. Hybridization was performed at 65°C in buffer containing 4× SSC, 10% dextran sulfate, 1× Denhardt solution, 5 mM EDTA, 0.1% Chaps, 50% deionized formamide, 200 μg/ml herring sperm DNA, 200 ng/ml DIG-labeled probe. The slides were then washed four times for 15 min each in 2× SSC and 0.1% SDS at 65°C and then thrice in 0.2× SSC and 0.1% SDS at 65°C and thrice with 0.2× SSC at room temperature. Colorimetric detection was performed using an anti-DIG antibody conjugated to alkaline phosphatase followed by incubation with NBT/BCIP color substrates using a digoxogenin-nucleic acid detection kit (Roche, Germany) as described previously. The results were assessed by light microscopy and digitally recorded. Positive signals appeared within 2 to 3 min of incubation with NBT/BCIP color substrate solution.

Results

We synthesized a polyclonal antibody against a conserved (identical in mouse and rat and conserved in human) amino terminal peptide (S45-K65 in mKCNA10) of KCNA10. To assess the specificity of the antibody, we tested its ability to recognize *in vitro* translated KCNA10 protein and distinguish it from Kv1.3 protein, the K channel protein most closely related to KCNA10 (17). Although anti-KCNA10 recognized a single band with an approximate molecular weight of 65 kd, which is nearly identical to that predicted by the primary sequence, it did not detect Kv1.3 protein (Figure 1a). Preim-

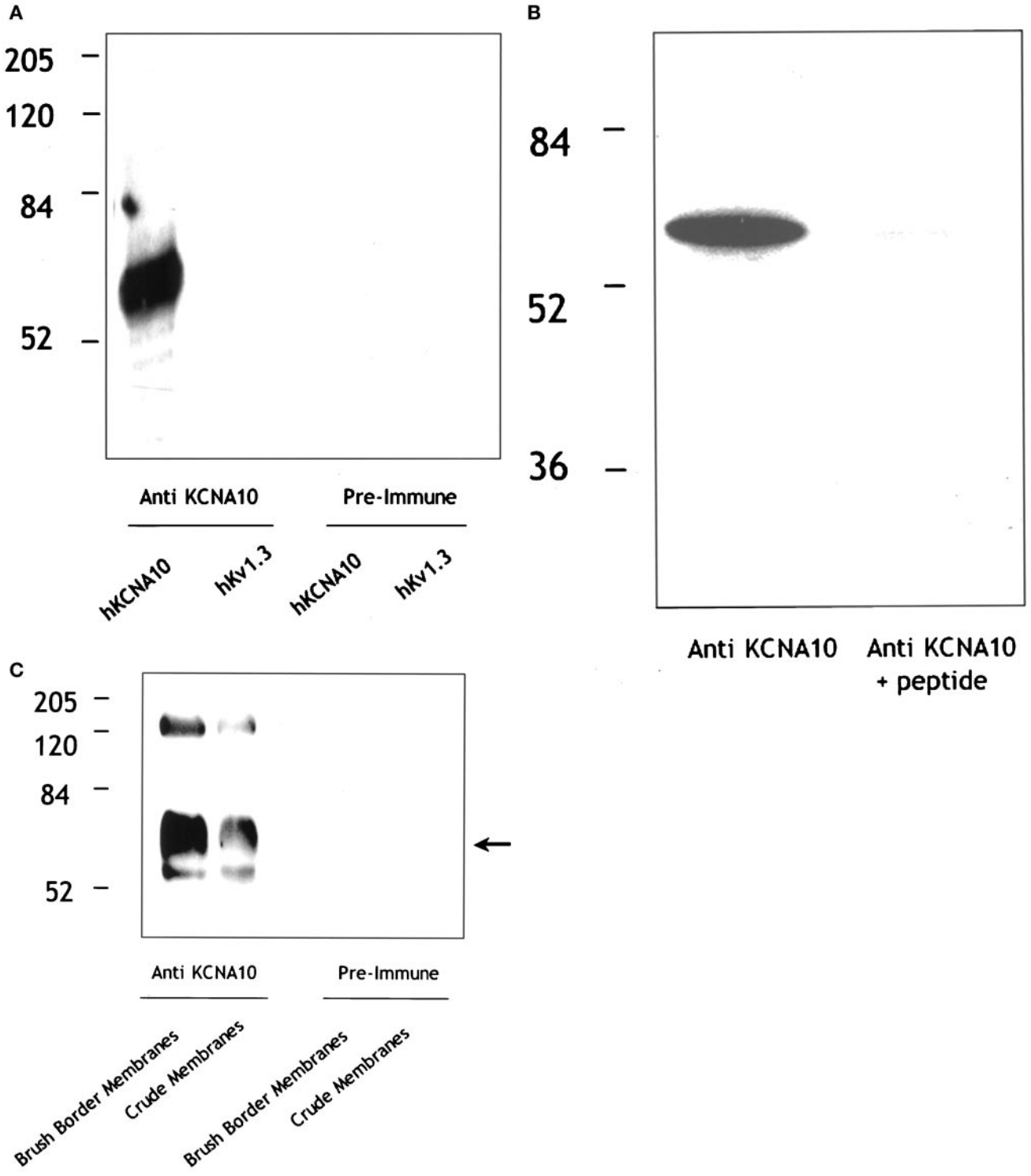

Figure 1. Specificity of a polyclonal antibody raised against KCNA10 protein. (A) Western blot showing that anti-KCNA10 labels *in vitro* translated hKCNA10 (approximately 65 kd) but not hKv1.3 (channel protein most closely related to KCNA10). Preimmune serum did not label any protein. (B) The anti-KCNA10 signal can be blocked by the antigenic peptide. Each lane contains 100 μg of crude rat renal membrane proteins separated by electrophoresis on a 10% SDS polyacrylamide. The lanes were cut out, and a Western blot was performed as described in Materials and Methods. The first lane (anti-KCNA10) was incubated with the primary antibody alone. The other lane (anti-KCNA10+ peptide) was incubated with anti-KCNA10 serum, which had been preincubated with the antigenic peptide. The signal detected by anti-KCNA10 crude renal membranes is of the expected size and is blocked by the antigenic peptide. (C) KCNA10 protein co-purifies with rat renal brush border membranes. Each lane contains 100 μg protein, and Western blotting is performed with the indicated primary antibody. KCNA10 (65-kd band) is relatively more abundant in brush border membranes than in crude membranes. The identity of the higher band is unclear and may represent KCNA10 protein complexes.

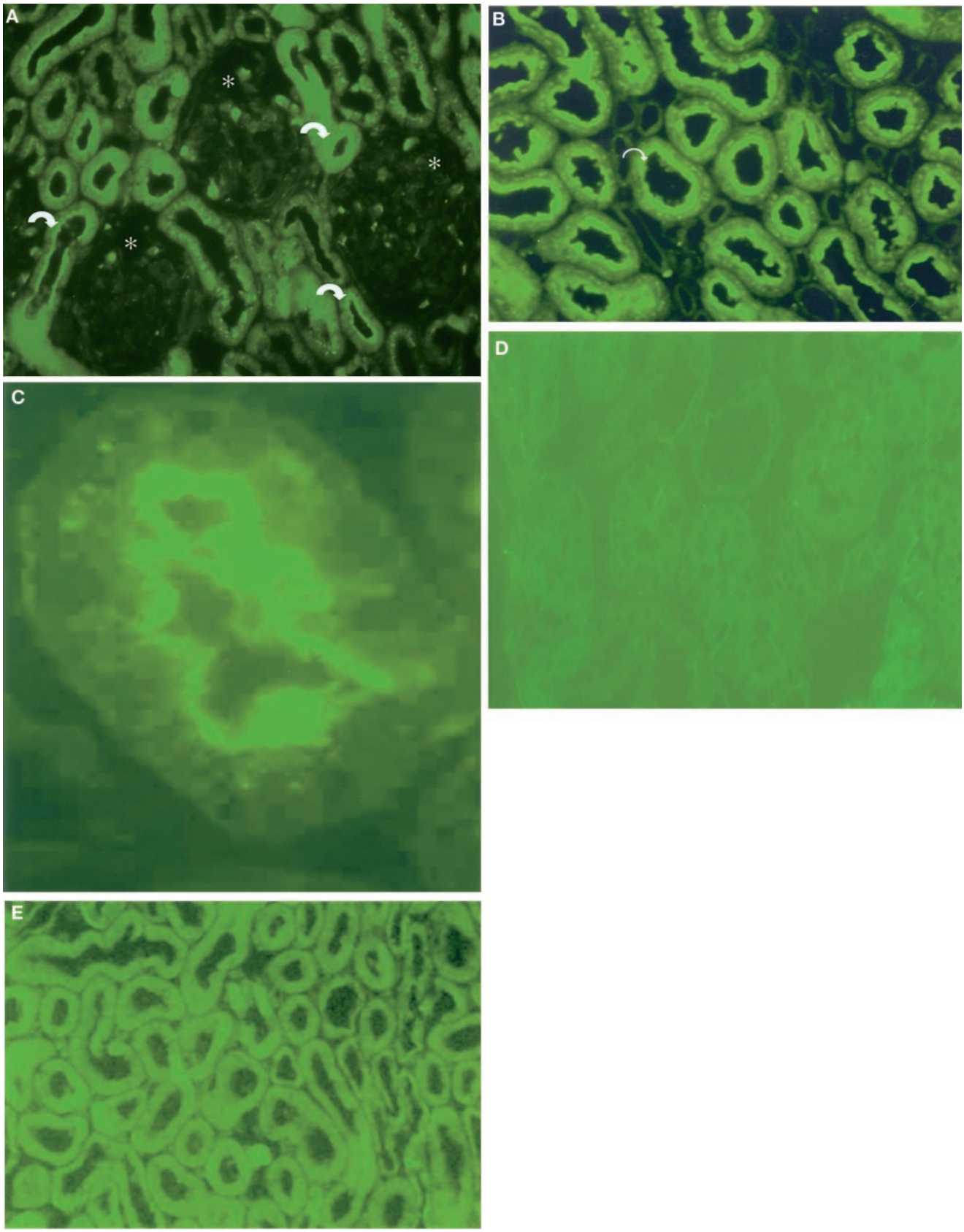

Figure 2. KCNA10 protein resides at the glomerulus and at the apical membrane of rat proximal tubular cells. (A) Renal expression KCNA10 protein expression was assessed by immunofluorescence. Anti-KCNA10 labeled only the glomerulus (*) and the apical membrane of proximal tubules (curved arrow). (B) Apical membrane labeling of proximal tubular cell. (C) Antigenic peptide blocks labeling by anti-KCNA10, indicating specificity of the signal. (D) Preimmune serum generates no signal. Magnifications: ×10 in A; ×20 in B, D, and E; ×40 in C.

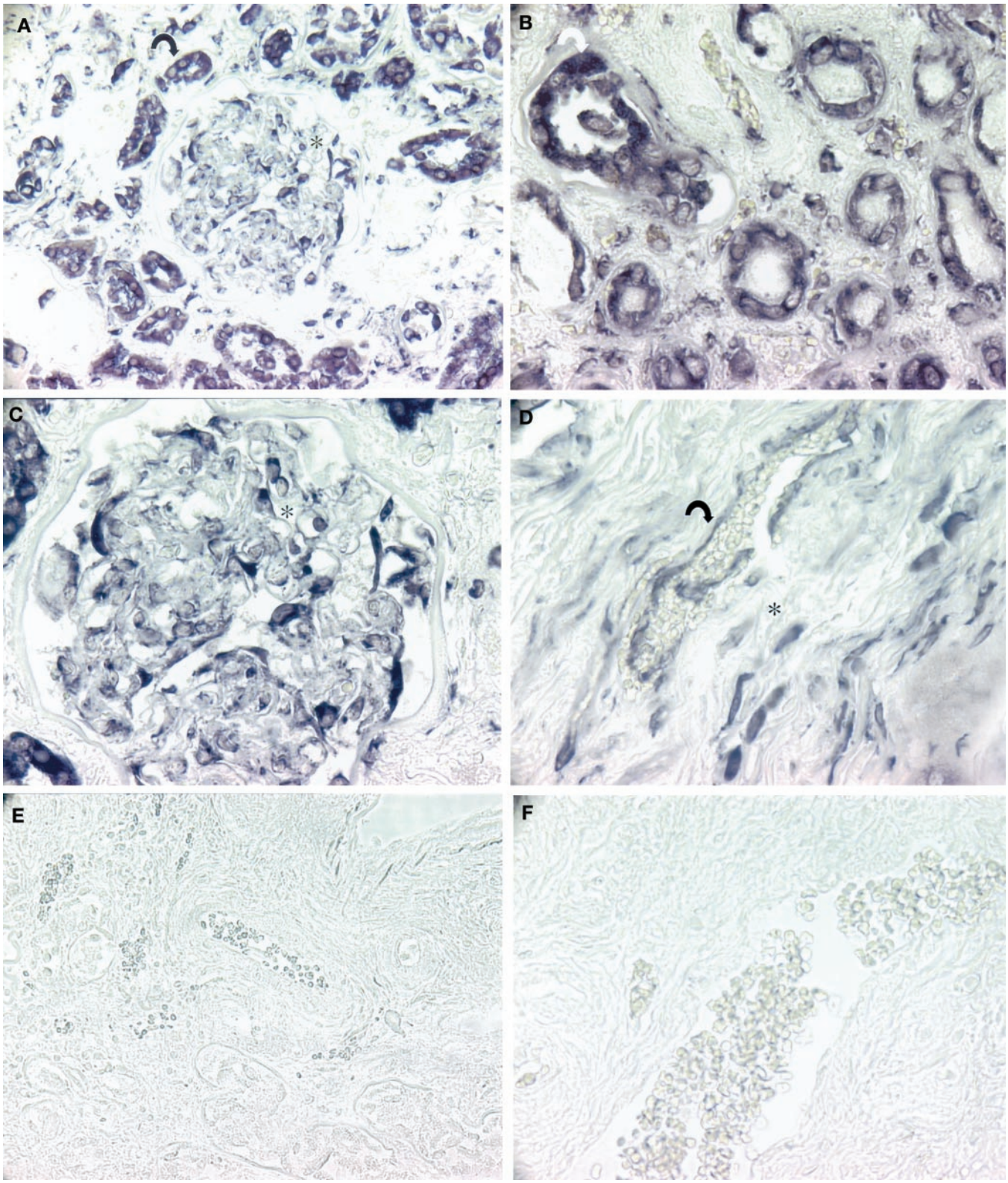

Figure 3. KCNA10 gene expression detected by *in situ* hybridization in human glomerular endothelilium (*) and proximal tubular cells (curved arrow). (A) Anti-sense probe. (B and C) Anti-sense probe. (D) Anti-sense probe labels endothelium (curved arrow) and smooth muscle cells (*) of intrarenal artery. (E) No signal detected in tubules and glomerulus with sense probe. (F) No signal detected renal vessels with sense probe. Magnifications: ×20 in A and E; ×40 in B, C, D, and F.

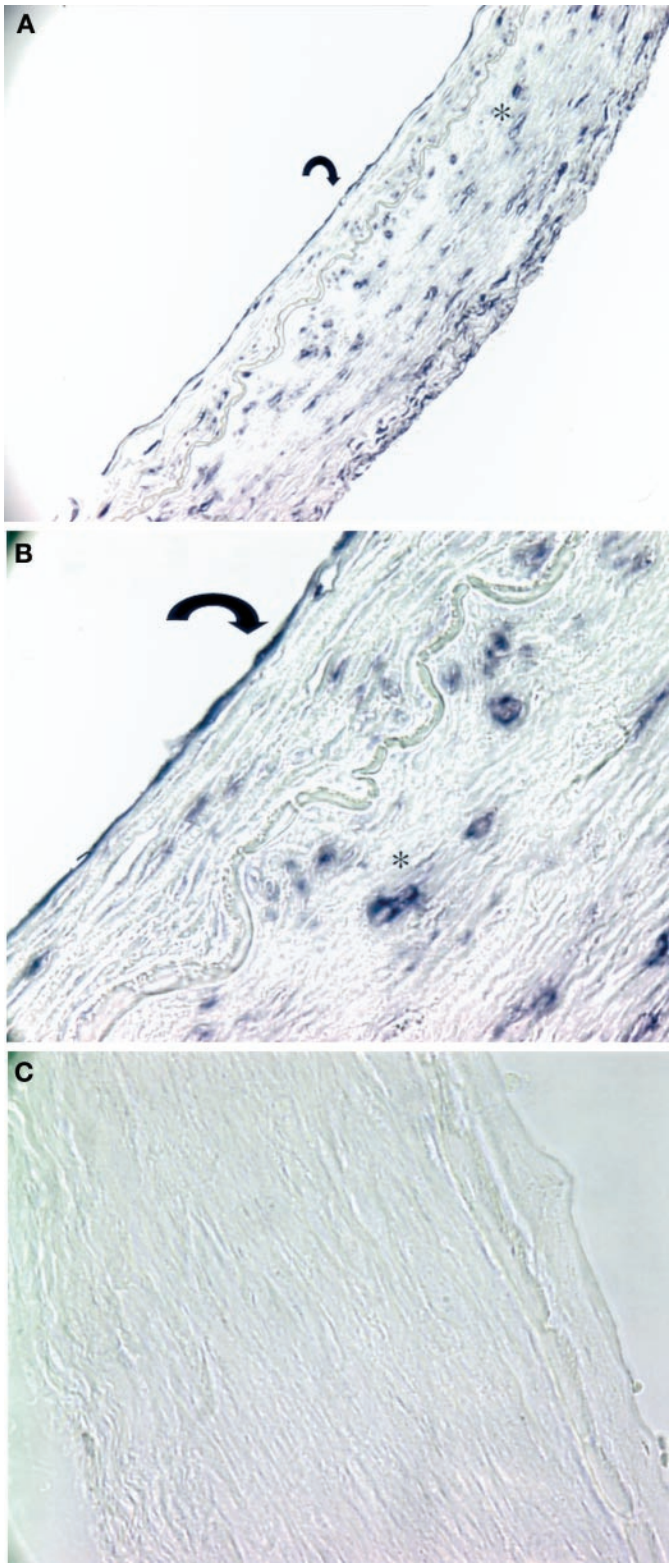

Figure 4. KCNA10 expression in human vascular endothelilium (curved arrow) and vascular smooth muscle cells (*). (A) *In situ* hybridization with antisense probe. (B) Antisense probe. (C) Sense probe detected no signal. Magnifications: ×10 in A; ×40 in B and C.

mune serum labeled neither KCNA10 nor Kv1.3 (Figure 1a). The specificity of the antibody was tested further in competition studies with the peptide used to generate anti-KCNA10. As shown in Figure 1b, the signal obtained with anti-KCNa10 was nearly completely abolished by the addition of the peptide. *KCNA10* is the only Kv channel gene that is more abundantly expressed in kidney than in brain (18). Figure 1c confirms that KCNA10 protein is highly expressed in kidney and readily detectable in both crude and purified membrane vesicles preparations. Brush border membranes, largely composed of apical membranes of proximal tubules, contain more KCNA10 protein than crude membranes, suggesting that KCNA10 may be a proximal tubular K channel.

The cellular localization of KCNA10 in rat kidney was determined by immunofluorescence. Anti-KCNA10 brightly labeled the apical membrane of proximal tubules, and a weaker signal was also evident at the glomerulus (Figure 2, a through c). The distal tubule and the limbs of Henle did not contain visible signals. Signal specificity was ascertained in competition studies (Figure 2d) and by using preimmune serum (Figure 2e). No labeling was detected in either study, confirming the specificity of the immunofluorescence signal observed with anti-KCNA10. These data corroborate the findings noted on Western Blot and indicate that KCNA10 is predominantly expressed at the apical membrane of the proximal tubule and at the glomerulus.

Although anti-KCNA10 detected hKCNA10 protein in Western blots (Figure 1, a and b), it was not useful for immunocytochemical studies, perhaps because the antigenic peptide is derived from the mouse sequence and only has 75% amino acid identity with human. Therefore, *in situ* hybridization was used to determine whether *KCNA10* expression in rat paralleled that in human. The anti-sense probe strongly labeled proximal tubular cells (Figure 3, a and b). A positive signal was also clearly evident in glomerular endothelial cells (Figure 3 a and c) and in intrarenal arteries (Figure 3d). Signal specificity is illustrated in Figures 3e and 3f, where the sense probe failed to generate a detectable signal. The *in situ* data are in agreement with those obtained by immunofluorescence and document KCNA10 expression in proximal tubular cells and in glomerular endothelial cells. To determine if KCNA10 was expressed in other blood vessels, we evaluated human cerebral artery by *in situ* hybridization. A positive signal was detected in both endothelial and vascular smooth muscle cells (Figure 4, a and b). Signal specificity was demonstrated using the sense probe (Figure 4c).

In summary, these data indicate that anti-KCNA10 specifically recognizes KCNA10 protein, which is expressed at the apical membrane of rat proximal tubule. *In situ* hybridization studies also demonstrate that *KCNA10* expression in rat kidney is similar to that in human.

Discussion

Role of KCNA10 in Renal Proximal Tubule

At first glance, the high level of expression of KCNA10 protein in proximal tubular cells, which are nonexcitable, is somewhat surprising. Indeed, the activation threshold of most Kv channels is >-60 mV and open only when the cell

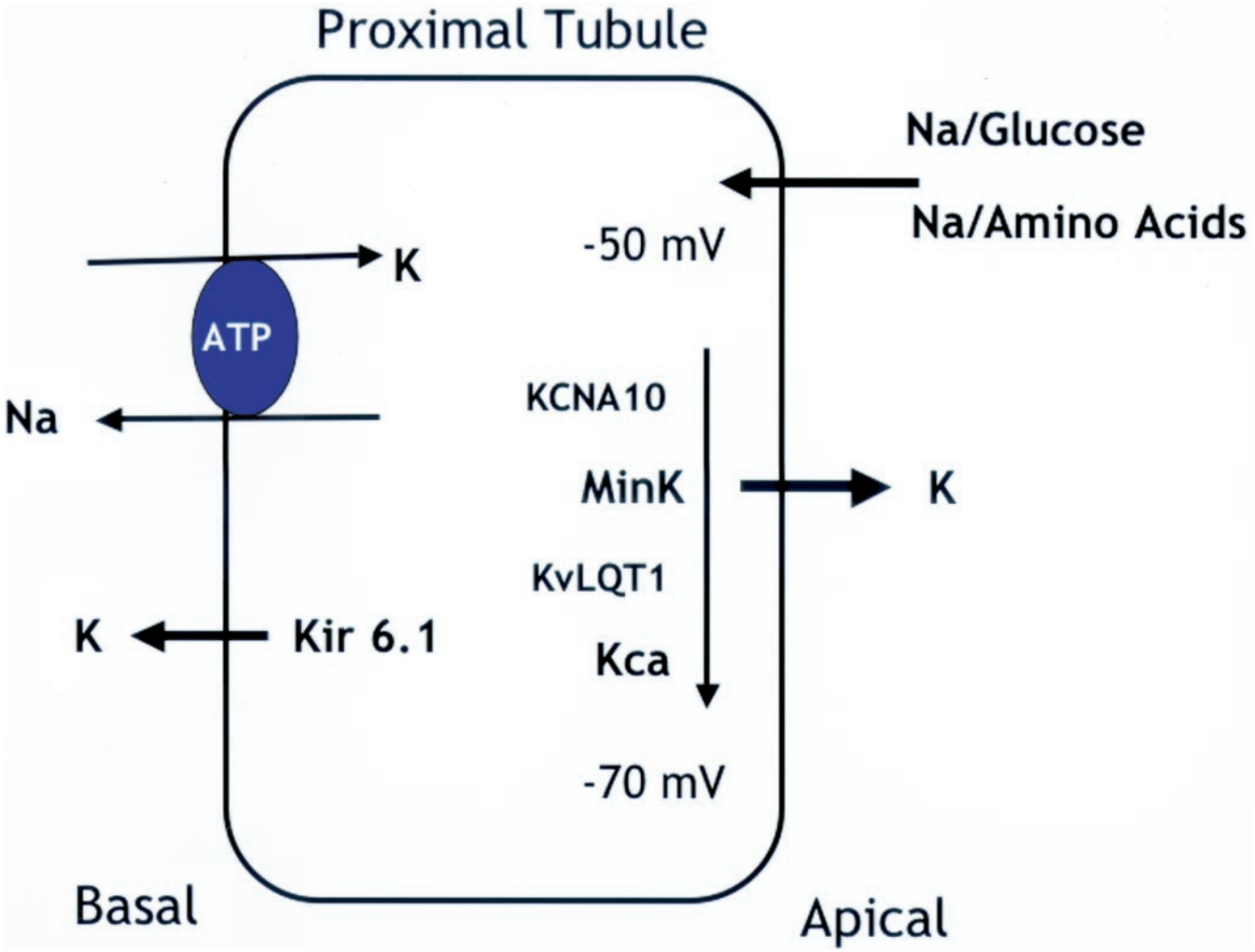

Figure 5. Proposed physiologic role of KCNA10 in the proximal tubule. Sodium entry depolarizes the cell membrane and K exit through KvLQT (Kv channel), KCNA10 and Kca (voltage and calcium gated K channel) at the apical and Kir 6.1 (inward rectifier) at the basolateral membrane repolarize the cell and maintain a favorable electrochemical gradient for Na, glucose, and amino acids absorption. MinK, accessory subunit of KvLQT1.

membrane depolarizes beyond −60 mV. Such channels would remain closed in many epithelial cells with resting membrane potential more negative than −60 mV under physiologic conditions. The proximal tubule contains several electrogenic transporters whose activities result in significant cell membrane voltage depolarization. Sodium entry is facilitated by a favorable electrical chemical gradient, and large changes in membrane potential have been recorded during sodium-coupled glucose and amino acid uptake. Proximal tubule cells have a resting membrane potential between −55 and −70 mV but also depolarize by 10 to 15 mV when electrogenic transport processes such as Na-glucose or Na-amino acid transport are activated (19). Depolarization of the cell membrane results in decreased in solute uptake; therefore, mechanisms that restore the membrane potential are critical. Kv channels, such as KCNA10 (activation treshold of −66 mV), are well suited for that task because they allow K efflux and cell membrane hyperpolarization when activated by membrane depolarization.

Kv channel activity has been detected at the apical membrane of proximal tubular cell using planar lipid bilayers (20). In patch clamp studies, the most commonly reported Kv channel belongs to the Maxi K class (21,22). In contrast, data obtained from Northern blotting, *in situ* hybridization, and immunocytochemistry clearly indicate the presence of at least two other types of voltage-gated potassium channels, namely KCNQ1 (23,24) and KCNA10. KCNQ1 forms a small conductance (2 to 10 pS) Kv channel and is thought to exist *in vivo* as a heteromultimeric complex with MInK, a small protein with a single transmembrane segment that is highly expressed at the apical membrane of the proximal tubule. Recent data indicate that KCNQ1 may play an important role in the regulation of sodium-coupled glucose and amino acid uptake, because mice deficient in MinK exhibit a reduction in glucose and amino acid uptake by the proximal tubule (25). These data confirmed the hypothesis put forward by Sugimoto *et al.* (26). The current work indicates that KCNA10 is also present at the apical membrane of the proximal tubule. By analogy with KCNQ1, we postulate KCNA10 may participate in stabilizing

membrane voltage during sodium entry at the apical membrane (Figure 5).

Role of KCNA10 in Glomerulus and Blood Vessels

Several types of K channels play a crucial role in the regulation of vascular smooth muscle contraction and, therefore, peripheral vascular resistance and BP (27,28). It appears that sustained arterial contraction is critically dependent on the interplay between transport pathways that mediate Ca^{2+} entry and extrusion. Ca^{2+} entry is primarily mediated by voltage-dependent Ca^{2+} channels and is therefore, regulated by changes in membrane voltage (29). Membrane hyperpolarization is mediated, in part, by the activation of K channels. Vasodilators modulate K channel activity in two ways. The K channel openers such as minoxidil, pinacidil, and diazoxide activate K channels by binding to the channel protein (30). Others, such as nitric oxide, nitroglycerin, nitroprusside, and isoproterenol, are thought to increase intracellular cGMP, which in turn activates K channels. KCNA10 is expressed in both endothelial (EC) and smooth muscle (SMC) cells, and its activity is regulated by cyclic nucleotides; therefore, it could participate in membrane repolarization and hyperpolarization.

In summary, KCNA10 is a cyclic nucleotide-gated Kv channel, expressed at the apical membrane of renal proximal tubular cells, well suited to stabilize cell membrane voltage during sustained Na entry. The channel is also present in glomerular and vascular endothelial cells and in vascular smooth muscle cells. We postulate that it regulates the membrane potential of these cells and contributes to the process of vasodilatation.

Acknowledgments

This study was supported by the Veteran Administration Merit Review award to Gary V. Desir and by NIH grant DK48105B.

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