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Primary structure and functional expression of a cGMP-gated potassium channel

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ABSTRACT **Cyclic nucleotides modulate potassium (K) channel activity in many cells and are thought to act indirectly by inducing channel protein phosphorylation. Herein we report the isolation from rabbit of a gene encoding a K channel (Kcn1) that is specifically activated by cGMP and not by cAMP. Analysis of the deduced amino acid sequence (725 amino acids) indicates that, in addition to a core region that is highly homologous to Shaker K channels, Kcn1 also contains a cysteine-rich region similar to that of ligand-gated ion channels and a cyclic nucleotide-binding region. Northern blot analysis detects gene expression in kidney, aorta, and brain. Kcn1 represents a class of K channels that may be specifically regulated by cGMP and could play an important role in mediating the effects of substances, such as nitric oxide, that increase intracellular cGMP.**

A large number of distinct potassium-selective (K) channels can be distinguished by electrophysiological methods. If one groups them according to modes of regulation, five broad categories emerge. Voltage-gated channels (K_V) open or close in response to changes in the membrane potential. Calcium-activated K channels (K_{CA}) are modulated by changes in the intracellular calcium concentration. Ligand-gated K channels (K_{LIG}) are controlled by changes in circulating hormone concentration. Nucleotide-gated K channels (K_{ATP} and K_{NUC}, respectively) respond to ATP and cyclic nucleotides. Stretch-activated K channels (K_{STR}) are controlled by mechanical forces sensed by the lipid bilayer. The molecular structures of K_V, K_{CA}, and K_{ATP} are known. Indeed, the Shaker gene family encodes K_V (1–5). The prototypic Shaker K channel has six transmembrane segments (TMSs) (S1–S6), a voltage-sensor (S4), and pore region contained between S5 and S6 (6). K_{CA} is encoded by the slowpoke gene (7, 8) and K_{ATP} belongs to the ROMK1/IRK family (9) of inward rectifiers. There is considerable interest in determining the structure of K_{NUC} since they may play important roles in the maintenance of arterial tone (10–12) and in the process of insulin secretion (13). In addition, a cGMP-gated voltage-independent K channel was recently detected by patch clamping the basolateral membrane of the rat cortical collecting duct (14, 15). That channel is postulated to play a critical role in determining the membrane potential of the basolateral membrane of the cortical collecting duct.

The molecular structure of a class of ion channels that are gated by cyclic nucleotides but do not discriminate between sodium and potassium (nonselective cation channels) is known (16–19). They all contain a cyclic nucleotide binding region located at the C terminus. These channels participate in both visual and olfactory signal transduction, a G-protein-dependent process in which cyclic nucleotides activate cation channels (20).

Sequence analysis indicates that cyclic nucleotide-gated cation channels may be distantly related to voltage-gated Shaker K channels since they possess a similar secondary structure and have amino acid homology limited to the voltage sensor (S4) and to the pore region (21). Furthermore, although the *Drosophila* ether a go-go (*eag*) gene (cAMP-activated, calcium- and potassium-selective channel) is more closely related to the cGMP-gated nonspecific cation channels (cGMP channel), it also has some similarities to Shaker K channels (22). Also, a K-channel protein, AKT1, recently isolated from the plant, *Arabidopsis thaliana*, contains a putative cyclic nucleotide binding region (35% sequence similarity to the cGMP binding domain of the rod cGMP-gated channel) (23) and homology to Shaker in the pore region.

Based on these data and on the fact that cyclic nucleotides are known to regulate K-channel activity (24), we hypothesized that K_{NUC} might share structural motifs with both Shaker K channels and cyclic nucleotide-gated cation channels and have isolated such a clone.†

MATERIAL AND METHODS

Library Screening and DNA Cloning. A rabbit genomic library cloned in EMBL-3 (Clontech) was screened using KC22, a partial-length Shaker-specific probe (25). KC22 was labeled by random-primer extension (0.5 to 1×10^9 cpm/μg of DNA) with [^{32}P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq; Amersham). A total of 1×10^6 clones were screened in duplicate at 42°C in buffer containing labeled probe at 0.5×10^6 cpm/ml, 50% (vol/vol) formamide, 0.5 M Na_2HPO_4, 1 mM EDTA, 7% (wt/vol) SDS, and 1% bovine serum albumin (pH 7.2). Filters were washed in 2× SSC (300 mM NaCl/30 mM sodium citrate, pH 7)/0.5% SDS at room temperature for 1 hr and then in 0.2% SSC/0.1% SDS at 42°C for 45 min. Twenty positive clones were identified. They were rescreened with a degenerate oligonucleotide primer [AA(C/T)AT(A/C)AA-(A/G)GG(I/C)AC(I/C)AA(A/C)ATGGG(I/C)AA(C/T)]. This primer is based on the amino acid sequence, NIKGSK-MGN, that is specific for the cGMP binding site of the bovine rod cGMP-gated cation channel (18). Screening conditions were the same as above except that formamide was omitted from the hybridization buffer and the final wash was at 42°C for 20 min. One cGMP-activated K channel clone (Kcn1/gen) was isolated and plaque-purified, and the genomic insert was excised with *Sac* I. Bands that hybridized to KC22 on Southern blot were cloned into pBluescript (Stratagene) and sequenced

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Abbreviations: *I–V*, current–voltage; TMS, transmembrane segment; K_V, K_{CA}, K_{ATP}, K_{NUC}, and Kcn, voltage-gated, calcium-activated, ATP-activated, cyclic nucleotide-activated, and cGMP-activated K channels, respectively.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. U38182).

by the dideoxynucleotide chain-termination method of Sanger *et al.* (26). Overlapping sequence of both strands was obtained by either subcloning the appropriate restriction fragments or by using sequence-specific oligonucleotides. Nucleotide and protein sequence analysis was carried out using the Genetics Computer Group (Madison, WI) software package on a VAX mainframe computer (Yale Biomedical Computer Center, New Haven, CT) and the program MACVECTOR (Kodak/IBI).

Phylogenetic tree analysis is based on amino acid sequence alignments using CLUSTALV (27) [Genetics Computer Group software package on VAX mainframe computer (Yale Biomedical Computer Center)]. The phylogenetic tree was reconstructed by using that alignment and a PAM250 residue weight table. The computer program MACVECTOR was used to align the sequences and to determine their secondary structure by the Robson–Garnier method (28).

Expression of Kcn1 in *Xenopus* Oocytes. Stage V–VI oocytes were dissected from ovarian lobes and stored in modified Barth's solution. The vegetal pole of selected oocytes was injected with 50 nl containing 5 ng of *in vitro*-transcribed 5′-capped Kcn1 RNA or water as a negative control. Whole cell currents were recorded by using a standard two-microelectrode voltage clamp. Voltage-clamped current measurements were made after a 1- to 3-day incubation period and expressed K currents were compared with those of water-injected control oocytes. For voltage clamping, oocytes were impaled with two microelectrodes filled with 3 M KCl (resistance, 0.5–5 MΩ).

Oocyte current–voltage (I–V) relationships were obtained by applying command voltage steps and measuring the resulting membrane current with a I–V amplifier (Warner Instruments, Hamden, CT). The command voltage square waves were held for a duration sufficient to achieve steady-state currents. In some experiments, a voltage ramp (0.4 mV/msec) from −100 to +100 mV was applied instead of steps. In either case, steps or ramps, similar I–V relationships were obtained. Currents were filtered at 2 kHz and the data were recorded and analyzed by using PCLAMP 5.1 (Axon Instruments, Burlingame, CA). The bath contained 88 mM KCl, 2 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 2.5 mM NaH_2CO_3, and 5 mM Hepes (pH 7.4). In experiments designed to measure the selectivity of the Kcn1 channel, external K was replaced with Na (K_{out} = 2 mM and Na_{out} = 88 mM). The pulse protocols are shown. To minimize ion fluxes prior to experimental maneuvers, the membrane voltage of the oocytes was clamped at a value close to the resting membrane potential. Therefore, when bathed in Na_{out} = 88 mM, the oocytes were clamped at a membrane voltage of −60 mV, and when K_{out} = 88 mM, they were usually held at 0 mV.

RESULTS AND DISCUSSION

We hypothesized that K_{NUC} might share structural motifs with both Shaker K channels and cyclic nucleotide-gated cation channels. Therefore, the following strategy was used to isolate

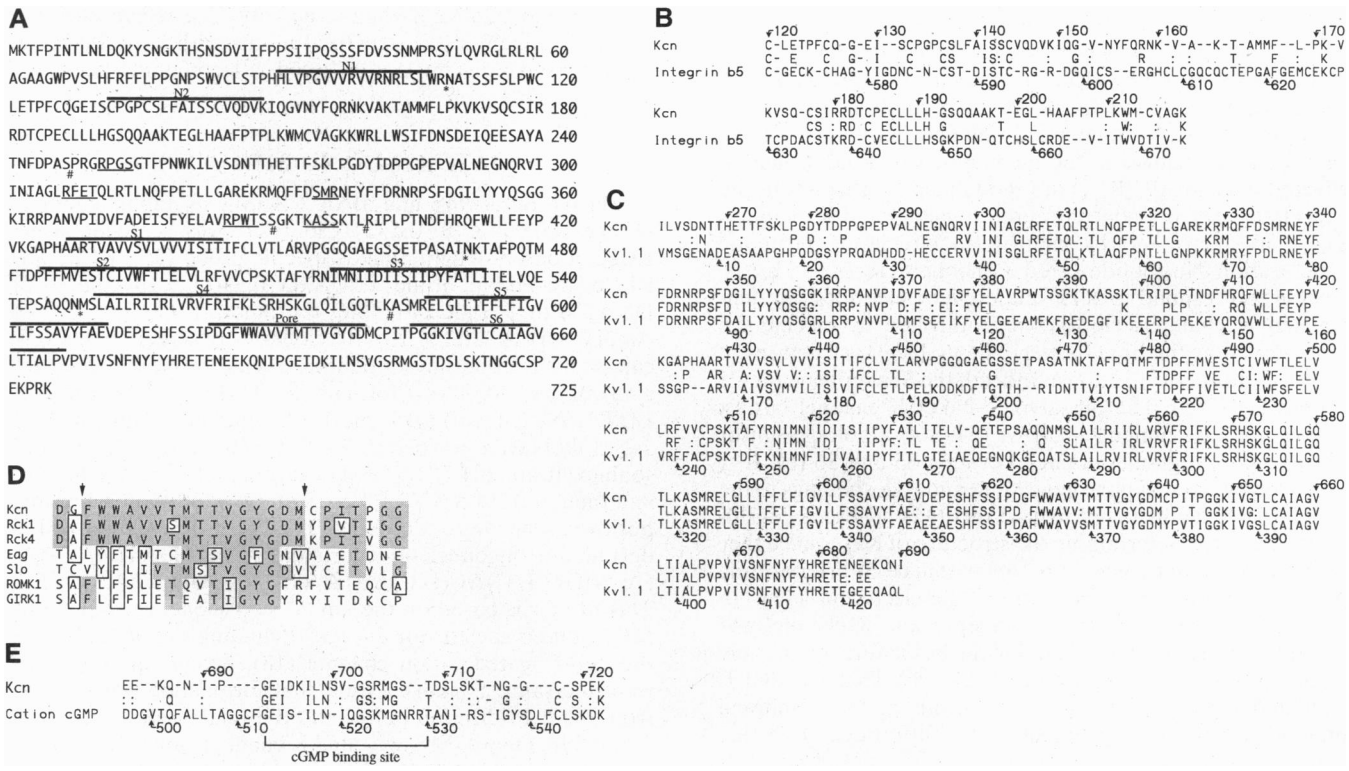

FIG. 1. (*A*) Deduced amino acid sequence of Kcn1. The deduced amino acid sequence for Kcn1 (725 aa) is shown. Transmembrane segments are labeled N1, N2, and S1–S6. Putative protein kinase A phosphorylation sites are underlined; protein kinase C sites are indicated as #; glycosylation sites are labeled with an asterisk. (*B*) The N terminus of Kcn1 contains a cysteine-rich region. Kcn1 (aa 120–220) is compared to integrin b5 (GenBank accession no. M35001). Gaps (dashes) have been introduced in the sequences for optimal alignment. Identical amino acids are shown as letters and conservative substitutions are shown as colons. The computer program ALIGN (DNAStar) was used to align the sequences. (*C*) Comparison of Kcn1 to Shaker Kv.1.1. Kcn1 (aa 260–690) is compared to Kv1.1 (1). Identical amino acids are shown as letters and conservative substitutions are shown as colons. Gaps (dashes) have been introduced in the sequences for optimal alignment. (*D*) Comparison of the K-channel pore region of Kcn1 to that of other K channels. Kcn1 is the reference sequence and is compared to RCK1 and RCK4, voltage-activated K channels from rat brain (29); Eag, cation channel protein (30); Slo, calcium-activated K channel (7); ROMK1, inward rectifier (31); and GIRK1, muscarinic K channel (32). Identical amino acids are shaded and conservative substitutions are boxed. The sequence delimited by the arrows represents the pore region that forms part of the ion conduction pathway in voltage-gated K channels. (*E*) Comparison of the cGMP binding domain of Kcn1 to that of cGMP-gated cation channels. Gaps (dashes) have been introduced in the sequences for optimal alignment. Identical amino acids are shown as letters and conservative substitutions are shown as colons. The cGMP binding site is predicted to form a β-sheet.

a putative cyclic nucleotide-gated K channel. A rabbit genomic DNA library was screened at moderate stringency with a Shaker K channel-specific probe. The positive clones were then rescreened with a degenerate oligonucleotide primer derived from the cGMP-binding region of the bovine rod cGMP-gated cation channel (18). A single 16-kb genomic clone (Kcn1/gen) that hybridized to both sets of probes was isolated.

The coding region (longest open reading frame) of Kcn1/gen is intronless and resides on a single exon. It encodes a 725-amino acid (aa) protein (Kcn1) with a predicted molecular mass of 81 kDa (Fig. 1*A*). Analysis of the deduced amino acid sequence reveals several important structural motifs. The N terminus (aa 80–220) contains a cysteine-rich region (10 cysteines) very similar to the "Cys-Cys loop" motif previously described in ligand-gated ion channels, ATP-activated cation channels (33), and the epithelial Na channel (34, 35). It is postulated that these cysteines could form disulfide bonds to stabilize a "ligand binding pocket" (33). It is also possible that such a region could mediate protein–protein interactions. Indeed, cysteine-rich domains are also present in the integrins, a class of cell adhesion molecules (36) and Kcn1 has a stretch of 22 aa (aa 177–198) that is highly homologous to integrin b5 (Fig. 1*B*).

Unlike the N terminus, Kcn1's central core region (aa 300–685) is homologous to members of the Shaker gene family. In that region, Kcn1 has 60–61% amino acid identity with the Shaker-related subfamilies Kv1.1, Kv1.2, and Kv1.3 (37, 38). Sequence similarity with Shaker-related proteins is greatest in the TMSs S4–S6 (Fig. 1*C*). The pore region of Kcn1 located between S5 and S6 is similar to that found in other previously cloned K channels as shown in Fig. 1*D*.

Sequence homology with Shaker K channels ends shortly after aa 685. The C terminus (56 aa) is 52% homologous (35% amino acid identity) to the cGMP binding region of cGMP-gated cation channels (Fig. 1*E*). In that region, both the amino acid sequence of the putative cGMP binding site (20 aa) and its predicted secondary structure are well conserved (70% amino acid homology and β-sheet structure). In particular, Thr-508 (cGMP channel numbering), thought to be critical for high-affinity cGMP binding (39), is conserved in Kcn1 (Thr-708).

Hydropathy analysis predicts that the core region of Kcn1 has a structure similar to that of Shaker proteins: six putative TMSs (S1–S6) and a pore region located between the S5 and S6. However, unlike Shaker the N terminus of Kcn1 contains at least two potential TMSs based on hydropathy analysis using both Kyte–Dolittle and Goldman–Engelman–Steitz scales with a 20-aa window (Fig. 2*A*). Since the exact number of TMSs in the N terminus is uncertain at present, two are shown in Fig. 2*B* in an attempt to satisfy the following constraints: the structure of the core region is similar to that of Shaker proteins and the N terminus is cytoplasmic (no evidence for leader peptide). Sequence analysis indicates that Kcn1 contains several putative phosphorylation sites for protein kinase A and protein kinase C and a single site (RPGSXXF) for cGMP-dependent protein kinase (40), suggesting that protein function may be regulated by phosphorylation.

Phylogenetic tree reconstruction (Fig. 2*C*) shows that Kcn1 shares a common ancestor with the Eag channel (Ca and K permeable) (30) and with the cyclic nucleotide-gated nonselective channels isolated from retina and olfactory epithelia. These results also indicate that Kcn1 is distinct from the known

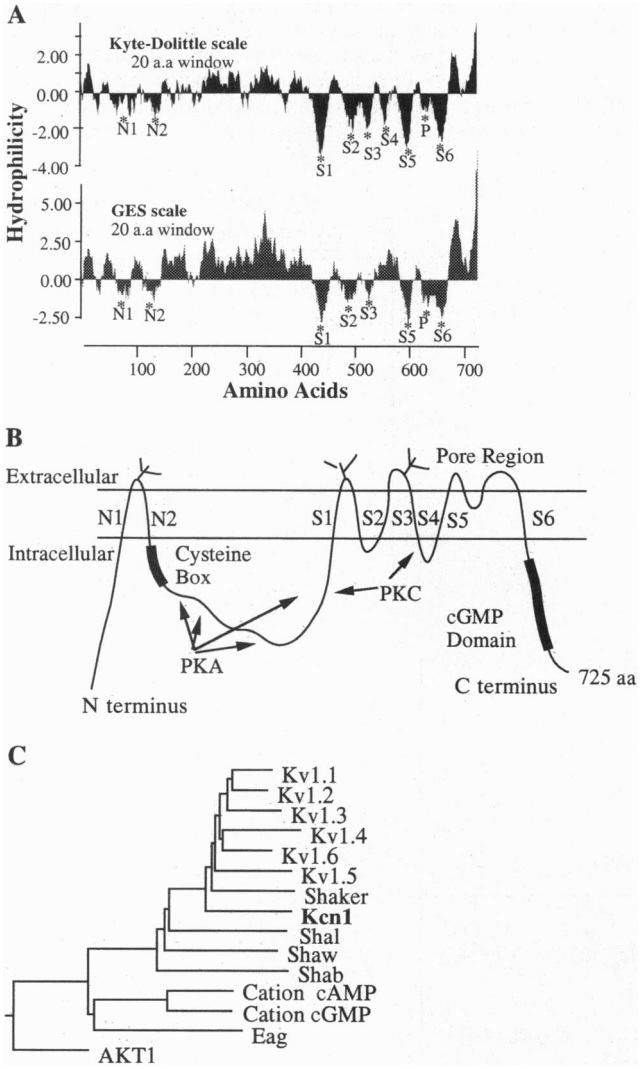

FIG. 2. (*A*) Hydropathy plot for Kcn1. Potential TMSs were identified by analyzing (MACVECTOR, IBI/Kodak) the deduced amino acid sequence with the Kyte–Dolittle and the Goldman–Engelman–Steitz (GES) scales with a 20-aa acid window. TMSs are indicated by an asterisk. (*B*) Proposed secondary structure model of Kcn1. TMSs are labeled N1, N2, S1–S6, and Pore. PKA, putative protein kinase A phosphorylation sites; PKC, putative protein kinase C phosphorylation sites. (*C*) Phylogenetic tree reconstruction of Kcn1. The length of each branch indicates the degree divergence from an ancestral node. Kv1–Kv1.6 represent prototypic members of the Shaker subfamily. Shaw, Shab, and Shal represent the other known members of the Shaker superfamily. Cation cAMP, cAMP-gated nonselective cation channel; cation cGMP, cGMP-gated nonselective cation channel; Eag, cation channel protein (22, 30); AKT1, K channel from plant (23). The GenBank accession numbers for the sequences used are as follows: Kv1.1, M30439; Kv1.2, M30440; Kv1.3, M30441; Kv1.4, X16002; Kv1.5, M27158; Kv1.6, M96688; Shaker, M17211; Shaw, M32661; Shab, M64228; Shal, S64320; cation cAMP, X55010; cation cGMP, X55519; Eag, M61157; AKT1, X62907; Kcn1, U38182.

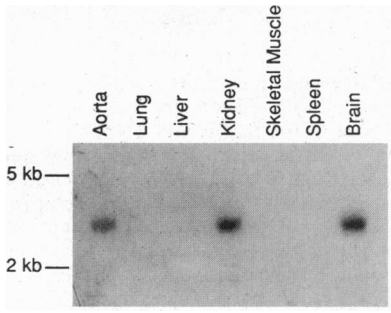

FIG. 3. Tissue distribution of Kcn1 gene expression. A probe consisting of the first 930 nt of the coding region was used to probe RNA isolated from the indicated tissues. Each lane contained 15 μg of poly(A)$^+$ RNA. Northern blot analysis was carried out as described (25).

Shaker-related genes. It is expressed in kidney, aorta, and brain but not in stomach, lung, liver, or spleen (Fig. 3).

To determine its kinetic properties, Kcn1 was expressed in *Xenopus* oocytes (41). Fig. 4*A* and *B* depicts typical *I–V* curves obtained with water (control) and RNA-injected oocytes. When bathed in 88 mM KCl/2 mM NaCl, oocytes injected with Kcn1 RNA exhibited both inward and outward noninactivating (300 msec) current with a peak of -0.6 ± 0.10 μA ($n = 22$) at -100 mV. Under similar conditions, peak current in water injected oocytes was -0.45 ± 0.06 μA ($n = 11$). The membrane-permeant cGMP analog 8-Br-cGMP was added to the bath and increased both inward and outward current in Kcn1-RNA injected oocytes (-0.6 ± 0.10 μA to -0.98 ± 0.15 μA at a membrane voltage $V_m = -100$ mV; $n = 22$). Unlike Shaker K currents, cGMP-induced K current was not activated by membrane depolarization. Although unexpected (Kcn1 has a well-conserved S4 region, the putative voltage sensor in Shaker channel), this is not unprecedented since the nonselective cGMP-gated cation channels are also voltage-independent in spite of having an S4-like region. Some Kcn1 RNA-injected oocytes showed inward rectification of the whole-cell K current (Fig. 4*A*). In contrast, 8-Br-cGMP did not affect current levels in water-injected controls ($n = 11$). Current activation by 8-Br-cGMP was evident within 5 min, peaked by 15 min, and was reversible with current levels returning to baseline within 10 min of washout. The addition of 8-Br cAMP to either control or Kcn1-injected oocytes did not change their current levels (Fig. 4*B*), suggesting that Kcn1 is specifically activated by cGMP. This finding is consistent with the fact that Kcn1 contains a conserved threonine residue necessary for high-affinity cGMP binding (aa 708, Fig. 1*E*). But it does not rule out the possibility that cGMP could modulate Kcn1 function by activating a cGMP-dependent protein kinase.

cGMP-activated current was detected during a narrow period (48–60 hr) after Kcn1 RNA injection and the current level

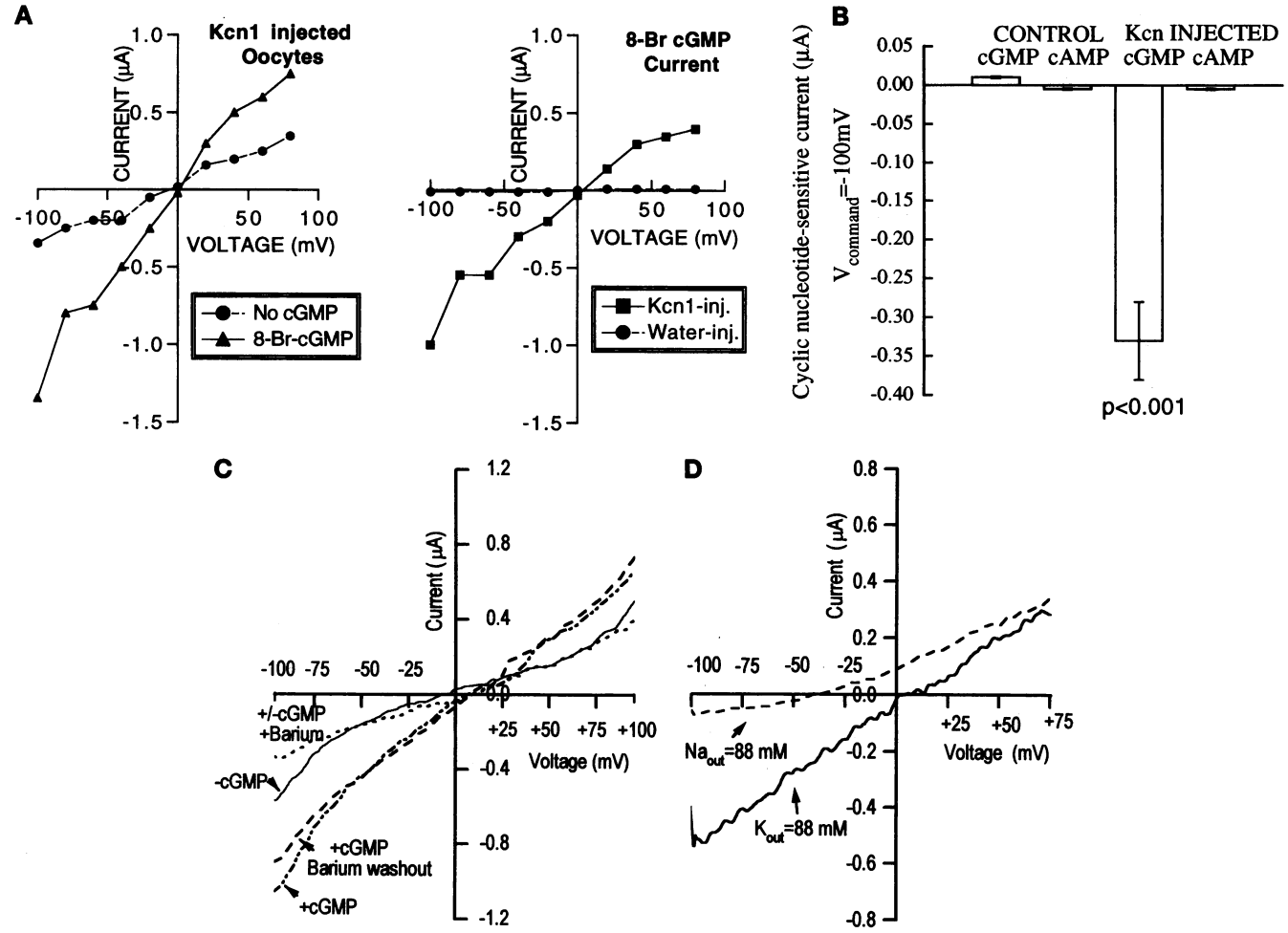

FIG. 4. cGMP-activated K currents in *Xenopus* oocytes injected with Kcn1 RNA. (*A*) Kcn1-injected oocytes express a cGMP-activated current. Oocytes were injected with either water (control) or 5 ng of Kcn1 RNA. Oocytes were bathed in 88 mM KCl/2 mM NaCl/1 mM $CaCl_2$/1 mM $MgCl_2$/2.5 mM NaH_2CO_3/5 mM Hepes, pH 7.4. Current tracings were obtained either by applying voltage steps from -100 to $+80$ mV in 20-mV increments from a holding potential of 0 mV or by applying a voltage ramp from -100 to $+100$ mV (0.4 mV/msec). In either case similar *I–V* relationships were obtained. Cyclic nucleotide-sensitive current was determined by subtracting current elicited before the addition of 2 mM 8-Br-cGMP from current measured after adding cyclic nucleotides. In preliminary studies, the rabbit reticulocyte lysate assay was used to show that the complementary RNA could direct the translation of a protein of the appropriate size (81 kDa). (*B*) cGMP specifically activated Kcn1 current. Cyclic nucleotide-sensitive current was determined by subtracting current elicited before the addition of cyclic nucleotides from current measured after adding cyclic nucleotides in oocytes bathed in 88 mM KCl and clamped at -100 mV. Oocytes were incubated with either 2 mM 8-Br-cGMP (control, $n = 12$; Kcn1 injected, $n = 22$) or 2 mM 8-Br-cAMP (control, $n = 5$; Kcn1 injected, $n = 5$) for 20 min and current tracings were obtained as described in *A*. (*C*) cGMP-activated current is barium-sensitive. Current tracings were obtained by applying a voltage ramp from -100 to $+100$ mV (0.4 mV/msec). The current tracings for each condition represent continuous ramps ($n = 10$) that have been averaged. Current activation is shown before and after the addition of 5 mM barium to the bath. Barium inhibited K current to a similar degree in the presence or absence of cGMP. For the sake of clarity only one curve is shown for these two experimental conditions. (*D*) cGMP-activated current is K-selective. External K^+ was replaced with Na^+ ($n = 7$; $Na^+_{out} = 88$ mM). Current tracings were obtained by applying a voltage ramp from -100 to $+75$ mV (0.4 mV/msec).

was low (0.33 μA at V_m = −100 mV). It may be that Kcn1 mRNA is not stable in *Xenopus* oocytes. We used different expression vectors and gene constructs and were unable to increase expression levels. The fact that Kcn1-induced cGMP-activated current did not exhibit a clear voltage dependence even though the putative voltage sensor S4 segment is well conserved suggests that voltage-gating may require additional elements. Indeed, Kcn1 may be part of a heteromultimeric complex and that one or more additional subunits are required for full expression of the cGMP-activated current. There is precedence for such a scheme as recently demonstrated for the epithelial Na channel (35) and for the nonselective cation channel from retina (42). In the case of the epithelial Na channel, expression in *Xenopus* oocytes of the subunit that was first isolated generated current sufficient to be detected by using the two-electrode voltage clamp but not by patch clamping (34).

External barium (5 mM) completely blocked cGMP-activated current (Fig. 4*C*), whereas tetraethylammonium (10 mM) had no effect. We observed both inward and outward cGMP-activated current with a reversal potential of 0.2 ± 5.2 mV when K_{out} was 88 mM (n = 12). When K_{out} was replaced with Na (88 mM), little inward cGMP-activated current was observed and the reversal potential shifted to −33.5 ± 4.8 mV (n = 7) (Fig. 4*D*). The data, when fitted to the Goldman–Hodgkin–Katz equation, assuming no Cl permeability, yielded a K/Na permeability ratio > 4:1. These results indicate that the Kcn1 expresses a cGMP-activated current that is K-selective and barium-sensitive.

cGMP-gated K channels have recently been detected in the basolateral membrane of the rat cortical collecting duct (14, 15). Although they have not yet been extensively characterized, it is known that their activity is critically dependent on the presence of cGMP and that they are voltage-independent and barium-sensitive. Thus far, Kcn1 is an excellent candidate for representing the molecular counterpart of these channels.

In conclusion, we have identified a gene encoding a K-selective channel (Kcn1) that is specifically activated by cGMP. This protein defines a class of K channels that has structural features common to voltage-gated Shaker-like K channels and to cyclic nucleotide-gated nonselective cation channels. In addition, it contains a cysteine-rich region previously noted in ligand-gated cation and the epithelial Na channels. We cannot rule out that Kcn1 associates with other subunits *in vivo* or that cGMP activates Kcn1 indirectly through a cGMP-dependent protein kinase. Since Kcn1 is activated by cGMP and expressed in vascular tissues, it could potentially participate in the regulation of arterial tone (43, 44).

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