Genomic structure and regulation of Kcn1, a cGMP-gated potassium channel

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Yao, Xiaoqiang, Yong Liu, Freeman Tung, and Gary V. Desir. Genomic structure and regulation of Kcn1, a cGMP-gated potassium channel. *Am. J. Physiol.* 271 (*Renal Fluid Electrolyte Physiol.* 40): F37–F41, 1996.—We recently cloned a novel rabbit gene that encodes a 725-amino acid protein (Kcn1) (Y. Yao, A. S. Segal, P. Welling, X. Zhang, C. M. McNicholas, D. Engel, E. L. Boulpaep, and G. Desir. *Proc. Natl. Acad. Sci. USA* 92: 11711–11715, 1995). Kcn1 RNA injected in *Xenopus* oocytes leads to the expression of potassium channels that are specifically activated by guanosine 3′,5′-cyclic monophosphate (cGMP). Northern blot and ribonuclease (RNase) protection analysis show that Kcn1 is differentially expressed in kidney, aorta, brain, and heart. The purpose of present study is to determine the structure of *Kcn1* gene, analyze the promoter region, and identify *cis*-regulatory elements responsible for transcription. We find that the coding region of Kcn is intronless. The major transcription initiation site was identified by primer extension. Sequence analysis of the 5′-flanking region indicates that, although the gene lacks a typical TATA box, it does have a TATA-box-like region (-TAT-). Using luciferase reporter constructs transfected in the porcine kidney cell line (LLC-PK_1), the promoter region and a 5′ enhancer element were identified by deletion analysis. Phorbol esters (12-*O*-tetradecanoylphorbol-13-acetate) and forskolin stimulated *Kcn1* gene expression 2.5- and 3.5-fold, respectively. In conclusion, we have identified the region of the novel potassium channel gene, *Kcn1*, that contains the promoter, a 5′ enhancer, and several *cis*-regulatory elements and shown that gene transcription is stimulated by cAMP and phorbol esters.

gene regulation; kidney; cyclic nucleotides; promoter analysis

CYCLIC NUCLEOTIDES MODULATE K^+ channel activity in a variety of mammalian cells (3, 5–7, 10, 13) and are thought to act indirectly by inducing channel protein phosphorylation. We recently isolated from rabbit a novel gene encoding a K^+ channel (*Kcn1*). Kcn1 channel activity in *Xenopus* oocytes is specifically increased by guanosine 3′,5′-cyclic monophosphate (cGMP) and not by cAMP (17). 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. These data indicate that Kcn1 represents a new class of K^+ channel that appears to be specifically regulated by cGMP. It could play an important role in mediating the effects of substances, such as nitric oxide, that increase intracellular cGMP. Northern analysis shows that its expression is tissue specific (kidney, aorta, heart, and brain). The purpose of this study is to take the first step toward elucidating the molecular basis of Kcn1 tissue-specific expression, that is to identify the *cis*-elements involved in gene transcription.

METHODS

Isolation of 5′-flanking sequences of Kcn1. A rabbit genomic library cloned in EMBL-3 (Clontech Industries) was screened using an 850-bp fragment of the coding region of KC22, a *Shaker*-related K^+ channel gene highly expressed in rabbit kidney (4). KC22 DNA was labeled by random primer extension ($0.5–1 \times 10^9$ cpm/μg DNA) with [^{32}P]dCTP (3,000 Ci/mmol, Amersham). A total of 1×10^6 clones were screened in duplicate at 42°C in buffer containing 0.5×10^6 cpm/ml of labeled probe, 50% formamide, 0.5 M Na_2HPO_4, 1 mM EDTA, 7% sodium dodecyl sulfate (SDS), and 1% bovine serum albumin, pH 7.2. Filters were washed in 2× SSC (300 mM NaCl, 30 mM sodium citrate, pH 7) and 0.5% SDS at room temperature for 1 h 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, NIKGSKMGN, that is specific for the cGMP binding site of the bovine rod cGMP-gated cation channel (9). 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 clone (Kcn1) was isolated, plaque purified, and the genomic insert was cut out with *Sac* I. Bands that hybridized to KC22 on Southern blot were cloned into pBluescript (Stratagene, San Diego, CA) and sequenced by the dideoxynucleotide chain termination method of Sanger et al. (14). Overlapping sequence of both strands was obtained by either subcloning the appropriate restriction fragments or by using sequence-specific oligonucleotides. The coding region of the gene was identified by Southern blotting and sequenced. One kilobase upstream to first ATG was then sequenced using the method of Sanger et al. (14).

Nucleotide and protein sequence analysis was carried out using the Genetics Computer Group software package on a VAX mainframe computer (Yale Biomedical Computer Center) and the program MacVector (Kodak, IBI).

Primer extension assay. A 30-nucleotide antisense primer (nucleotides 490–519 of coding sequence) was synthesized in vitro and end-labeled with ^{32}P using T4 polynucleotide kinase. The hybridization reaction contained 10 μg of poly(A)$^+$ RNA in 80% formamide, 1 mM EDTA, 0.4 M NaCl, 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), pH 6.4, and 1×10^6 cpm of labeled probe in a total volume of 30 μl. The mixture was heated to 85°C for 10 min and then incubated overnight at 30°C. The RNA-probe complex was precipitated and resuspended in 20 μl of hybridization buffer. The primer was then extended by adding 200 U of Moloney murine leukemia virus reverse transcriptase (SuperScript) and incubating for 2 h at 37°C. The reaction was stopped by adding 1 μl of 0.5 M EDTA. The mixture was digested with 1 μl of ribonuclease A (RNase A; 5 μg/ml stock) and incubated at 37°C for 30 min. The reaction was extracted with phenol-chloroform, and the products were size fractionated by denaturing polyacrylamide (5%) gel electrophoresis (20). *Hae* III digest fragments of λ-bacteriophage were end labeled with [γ-^{32}P]ATP using T4 kinase (Boehringer). The gel was dried

and exposed to film for 16 h with 1 intensifying screen at −50°C. Fragment size was determined by computer (Visage 2000, Bio-Image) and by comparison to a sequencing reaction of a known template.

Plasmid construction. A 1.1-kb fragment [−731/+357; 731 bp upstream to 357 downstream of the transcription initiation site (TIS)] was amplified by polymerase chain reaction (PCR) with the following primers: sense, GCT AGC TCC AGG GGC AGG TCC TGG GC; antisense, AGA TCT TGG GGT GGA TAG ACA AAC CC (*Nhe* I and *Bgl* II sites are added at the 5′ end of the sense and antisense primers, respectively). PCR was carried out using an Air Thermo-Cycler (Idaho Technology) as follows: denature at 94°C for 5 s, anneal at 55°C for 10 s, and extend at 72°C for 60 s, 40 cycles. The PCR product was cut with *Nhe* I and *Bgl* II and directionally cloned (to maintain 5′-3′ orientation) into the Basic Genelight (Promega) plasmid also cut with *Nhe* I and *Bgl* II. The Basic Genelight plasmid is a reporter vector that encodes luciferase and that contains neither a promoter nor an enhancer. Identity and orientation of the 1.1-kb fragment was confirmed by Sanger sequencing. Progressive deletion (deletion mutants) of the 5′ end of the 1.1-kb fragment was achieved using Exonuclease III (Erase a Base system, Promega) according to the manufacturer's protocol.

Several other DNA fragments (−731/−192, −193/+852, −88/+97, −88/+357, and +98/+357) were used. Fragment −731/−192 was amplified by PCR and cloned (both 5′→3′ and 3′→5′ directions) into Basic Genelight (no enhancer, no promoter) and Promoter Genelight (contains SV40 promoter but no enhancer). All other fragments were subcloned (5′-3′ direction) into Basic Genelight Vector. The identity and orientation of all constructs were verified by Sanger sequencing.

Cell transfection and luciferase assay. LLC-PK_1 cells were grown in 80% α-minimal essential medium (α-MEM) and 20% fetal bovine serum (FBS) at 37°C to 70% confluence in 35-mm tissue culture dishes. One hundred microliters of Opti-MEM Reduced Serum medium containing 1.7 μg of the plasmid of interest were mixed with 7.5 μl of LipofecAmine (GIBCO-BRL) diluted into 100 μl Opti-MEM Reduced Serum medium in a 5 ml polystyrene tube. The mixture was incubated at room temperature for 45 min. The cells were washed twice with 1 ml of Opti-MEM Reduced Serum medium. Transfection was then initiated by adding 0.8 ml of Opti-MEM Reduced Serum medium and 0.2 ml of the plasmid/Lipofect-Amine mixture to the cells. After a 6-h incubation, 1 ml of culture medium (80% α-MEM and 20% FBS) was added, and the mixture was incubated for 12–16 h at 37°C. The culture medium was then changed to α-MEM with 10% FBS, and the cells were incubated for an additional 24 h. When indicated, 25 μM forskolin or 0.1 μM phorbol ester [12-*O*-tetradecanoylphorbol-13-acetate (TPA)] was added prior to the 24-h incubation.

Luciferase production was then measured as follows. The cells were washed twice with 2 ml of phosphate-buffered saline and then lysed by adding 150 μl of lysis buffer (Luciferase Assay Kit, Promega) and incubated for 15 min at room temperature. The cell lysate was collected and stored at −20°C and assayed within 2 days. Ten microliters of cell lysate were added to 100 μl buffer containing ATP and luciferin (Promega), and bioluminescence was measured within 30 s by liquid scintillation.

The optimum time course for measuring luciferase production was determined empirically in preliminary studies. A control plasmid was not used to correct for transfection efficiency because we found, as others have (16), that the addition of the control plasmid significantly reduced transfection levels of the plasmid of interest. Instead, experiments were carried out in triplicate and repeated at least five times.

RNase protection assay. The cDNA template was generated by amplification of the region of interest in Kcn1 (nucleotides +1/+300 of the coding region) using PCR driven by gene-specific primers. The antisense primer used contained the consensus sequence for T7 RNA polymerase (30 bp). The labeled antisense riboprobe was synthesized directly from PCR-amplified cDNA using T7 RNA polymerase (Promega, Biotec) and [^{32}P]UTP (3,000 Ci/mmol, Amersham) (protocol provided by Promega) and was gel purified (5% polyacrylamide). The hybridization reaction contained 10–100 μg of total RNA (isolated from freshly dissected rabbit organs) in 80% formamide, 1 mM EDTA, 0.4 M NaCl, 40 mM PIPES, pH 6.4, and 2.5 × 10^5 cpm of antisense riboprobe in a total volume of 30 μl. The reaction mixture was overlaid with 50 μl of mineral oil and incubated at 50°C for 16 h. The total amount of RNA in each sample was kept constant (100 μg) by adding the appropriate amount of yeast tRNA. The hybridization products were digested by adding 300 μl of an RNase digestion mixture [300 mM NaCl, 5 mM EDTA, 10 mM tris(hydroxymethyl)aminomethane, pH 7.4, 2 μg/ml RNase T1, and 40 μg/ml RNase A] and incubating for 1 h at 32°C. The mixture was then incubated for 30 min at 37°C with proteinase K (0.03 mg/ml) in the presence of 0.6% SDS. The reaction was extracted with phenol-chloroform, and the RNase-resistant products were precipitated by adding 1 μg of glycogen and 750 μl of ice-cold ethanol and incubating at −70°C for 1 h. The pellet was washed with 70% ethanol, dried at room temperature, and dissolved in formamide loading buffer (80% formamide, 10 mM EDTA, pH 8, 1 mg/ml xylene cyanol FF, and 1 mg/ml bromophenol blue). The reaction products were size fractionated by denaturing polyacrylamide (5%) gel electrophoresis (15). *Hae* III digest fragments of λ-bacteriophage were end labeled with T4 polynucleotide kinase (Boehringer) and [γ^{32}P]ATP and used as size markers. The gel was dried and exposed to film for 48 h with two intensifying screens, at −50°C. Since the intact probe contains ~30 bp of nonhomologous sequence, it is, therefore, easily distinguished from a protected fragment.

RESULTS AND DISCUSSION

Identification of a 5′-flanking region with promoter activity. Sequence analysis of the coding region of *Kcn1* from the genomic clone indicates that it is intronless and that the longest open-reading frame (ORF) resides on a single exon. This is confirmed by the fact that amplification of that region (2,300 bp) from genomic DNA and kidney cDNA by PCR yields products of identical size. Several *Shaker*-like voltage-gated K^+ channels are reported to be intronless in vertebrates but not in *Drosophila* (2).

Primer extension analysis shows that the *Kcn1* gene has one major transcription initiation site (Fig. 1) ~90 bp upstream of the beginning of the longest ORF. We tested a 1.1-kb region (731 bp upstream and 357 downstream of the TIS) for transcriptional activity. This fragment was amplified by PCR and cloned into the Basic Genelight reporter vector (lacks promoter and enhancer), which contains the gene for firefly luciferase. The construct was transiently expressed into the renal cell line LLC-PK_1. As shown in Fig. 2, the 1.1-kb fragment was capable of driving gene transcrip-

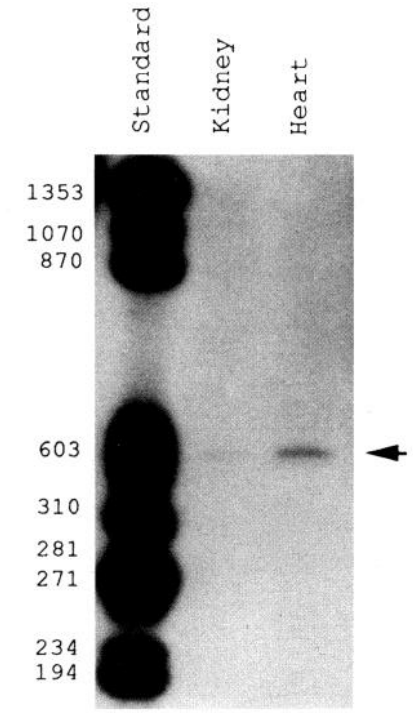

Fig. 1. Localization of the transcription initiation sites (TIS). TIS were localized by primer extension. The number of base pairs is indicated on *left*. Ten micrograms of poly(A)$^+$ RNA were used for the reaction. Based on the location of the primer and preference of TIS for nucleotide -CA-, we deduced that the major TIS is located 88 nucleotides upstream of the longest open-reading frame (ORF).

tion when cloned in a promoterless Genelight plasmid, indicating that it contains a functional promoter.

Localization of the promoter and of a 5′ enhancer region. Progressive 5′ deletion of the 1.1-kb fragment was carried out using Exonuclease III. Deletion of the first 50 or 150 bp from the 5′ end of the 1.1-kb fragment significantly reduced transcriptional activity (Fig. 2),

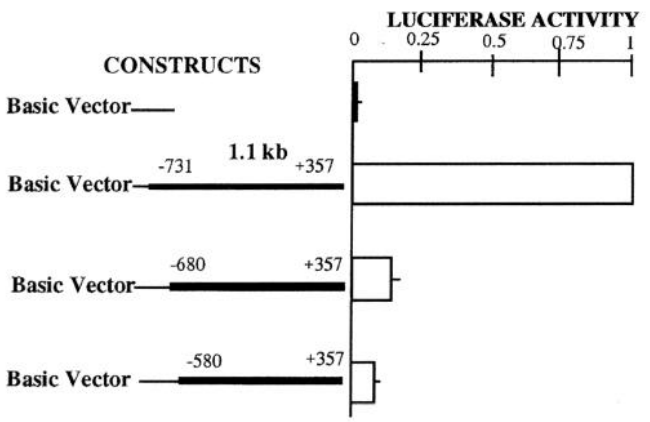

Fig. 2. Isolation of a transcriptionally active 5′-flanking region in the *Kcn1* gene. A 1.1-kb 5′ fragment (−731/+357) was cloned into a promoterless (Basic) reporter vector. The construct was transfected into LLC-PK$_1$ cells, and luciferase activity was measured as described in METHODS. For a negative control, the Basic vector without insert is transfected. Deletion mutants (−680/+357 and −580/+357) were generated using Exonuclease III. Deletion fragments were cloned into the promoterless (Basic) reporter vector. Activity of the Basic vector with the 1.1-kb insert was normalized to 1. All experiments were carried in triplicate and repeated 4 times.

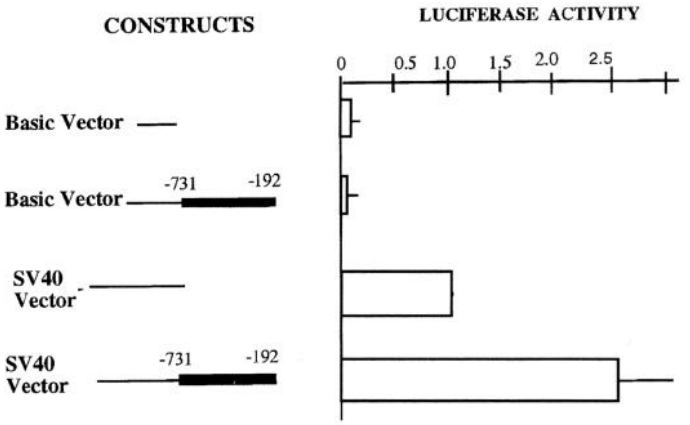

Fig. 3. Identification of 5′ enhancer in the *Kcn1* gene. Fragment −731/−192 derived from 5′ end of the 1.1-kb sequence was cloned in a promoterless (Basic) vector or a promoter vector (SV40 vector). Transcriptional activity was measured by the luciferase assay. For a negative control, the Basic vector without insert was transfected. All experiments were carried in triplicate and repeated five times. Activity of promoter vector was normalized to 1. All experiments were carried in triplicate and repeated 4 times.

indicating that this small region was critical and contained either the promoter or a strong enhancer element.

To distinguish between these two possibilities we tested whether the 5′ end of the 1.1-kb fragment (−731/−192) could support transcription in a promoterless (Basic) vector. It did not, suggesting that this region is actually an enhancer element (Fig. 3). This was confirmed by showing that, although the 5′ fragment (−731/−192) had no intrinsic transcriptional activity, it enhanced baseline activity of a heterologous promoter (Fig. 3). Furthermore, transcriptional activation was orientation independent and the fragment still enhanced activity of the SV40 promoter when cloned in the reverse direction (data not shown). These results indicate that a strong enhancer element is located between −731 and −192. To localize the position of promoter, we tested the promoter activities of various constructs. The data shown in Fig. 4 indicate that the constructs −192/+852, −88/+357, and −88/+97 all have promoter activities. Since these constructs all

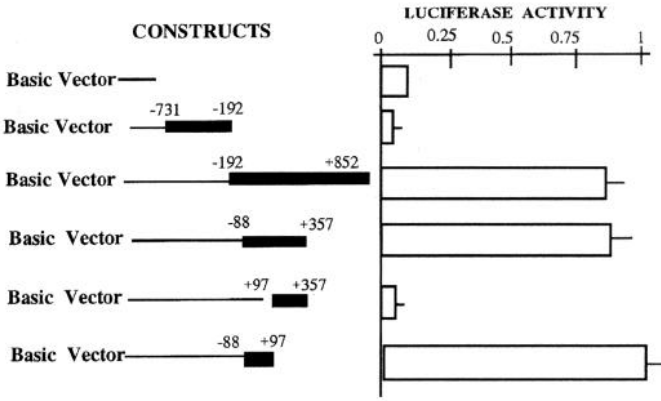

Fig. 4. Minimal promoter of the *Kcn1* gene. Fragments are cloned in a promoterless (Basic) plasmid. Activity of basic vector with inserts (−88 bp/+97 bp) was normalized to 1. All experiments were carried in triplicate and repeated 4 times.

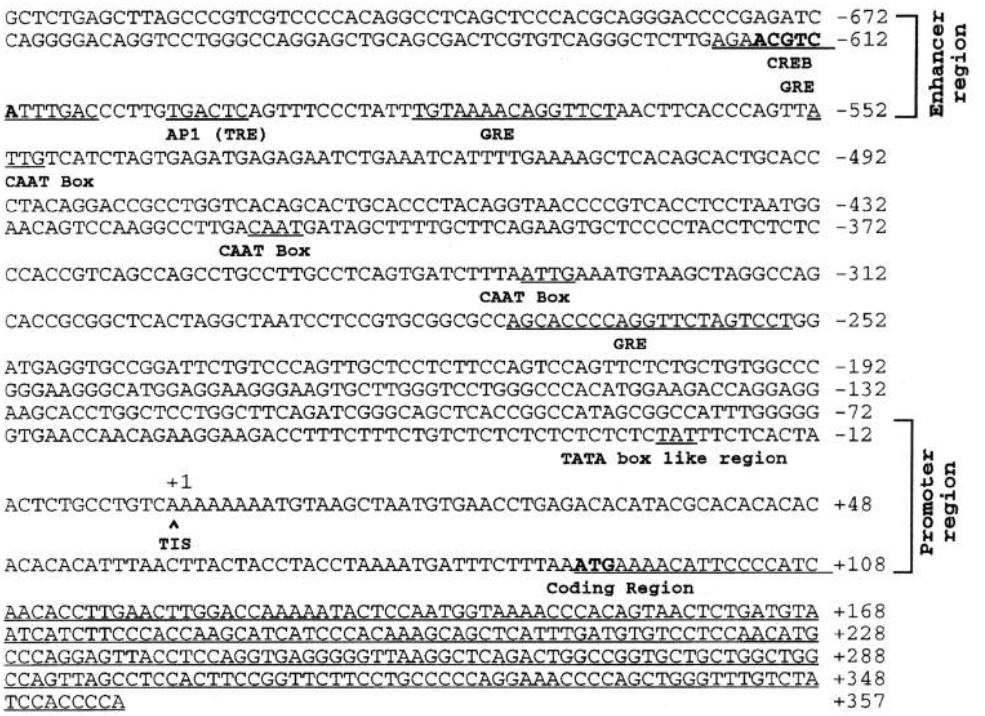

Fig. 5. Sequence of 5′-flanking region of the *Kcn1* gene. Nucleotide sequence of 5′-flanking sequence of the *Kcn1* gene. Sequencing of both strands was carried out using the method of Sanger et al. (14). TATA-box-like region, CAAT box. GRE, glucocorticoid responsive element. CREB, cAMP responsive element. AP1, activator protein 1 binding site; TRE, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) responsive element.

contain a −88/+97 region, we conclude that the minimal promoter is located between −88/+97.

Sequence analysis of 5′-flanking region. The 731-bp region upstream of the TIS was sequenced (Fig. 5). Examination of the nucleotide sequence reveals that, although *Kcn1* lacks a typical TATA box, it has a TATA-box-like region (-TAT-) 24 bp upstream of the TIS. The major TIS is located ~90 bp upstream of the longest ORF (Fig. 1). That region contains a consensus sequence of a eukaryotic cap site (TCA) (1). *Kcn1* is not a housekeeping gene (which often lacks a TATA box), since, as shown in Fig. 6, it is differentially expressed with message detectable in kidney, heart, aorta, brain, and muscle but absent from liver, stomach, and spleen. Additionally, *Kcn1* does not have Sp1 sites, which are usually found in housekeeping genes.

Regulation of Kcn1 gene transcription by cAMP and phorbol esters (TPA). Consensus sequences for steroid hormone, cAMP (CRE), and phorbol esters (TRE) response elements were identified in the 731-bp 5′-flanking region. We next determined whether the CRE and TRE regions identified in the region were functionally active. To that end, the effects of TPA and forskolin on *Kcn1* transient gene expression in LLC-PK_1 were examined. Cells were transfected with the 1.1-kb fragment cloned into the Basic Genelight plasmid and then treated with either the phorbol ester TPA or forskolin (increases cAMP) for 24 h. As shown in Fig. 7, the 1.1-kb fragment had significant transcriptional activity that was further enhanced by treatment with either TPA (260 ± 30%; $n = 22$, $P < 0.001$) or forskolin (349 ± 57%; $n = 14$, $P < 0.001$). Voltage-gated K channel gene expression (Kv1.5) has been shown to be regulated by cAMP in a cell-specific manner by both CRE-binding and CRE-modulator proteins (12). We are currently testing whether Kcn1 is regulated in a similar manner. Our data are consistent with the hypothesis that the TRE and CRE regions identified in the *Kcn1* gene mediate transcriptional activation. However, they do not prove that these regions are the only or the major sites of regulation. Mutational analysis of these sites will provide a more detailed understanding of their importance in *Kcn1* gene regulation. Nevertheless, it is clear that cAMP and phorbol esters activate *Kcn1* gene transcription in vitro.

The 5′-flanking region of the *Kcn1* gene also contains a consensus sequence for a previously identified composite glucocorticoid response element (plfG) (8, 11). The interaction of specific transcription factors at plfG may determine whether mineralocorticoid or glucocorticoids bind to it and whether binding of glucocorticoids result in gene activation or inhibition. In preliminary studies,

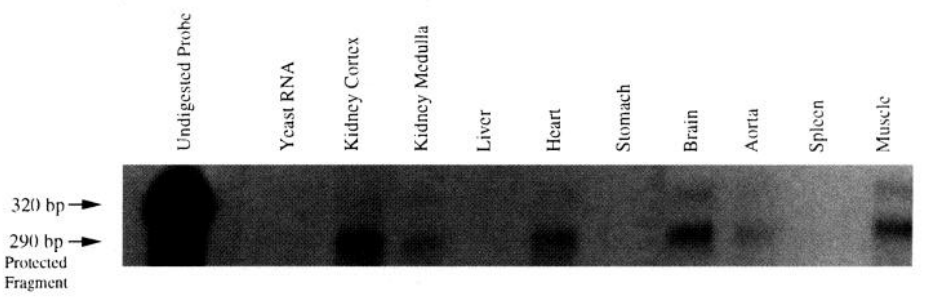

Fig. 6. Differential expression of the *Kcn1* gene in the rabbit RNase protection assays was carried out as described in METHODS. A quantity of 100 μg of total RNA was used in each reaction. As control, each probe was also hybridized to 100 μg of yeast tRNA. Bands represent protected fragments, and intensity indicates the relative transcript abundance.

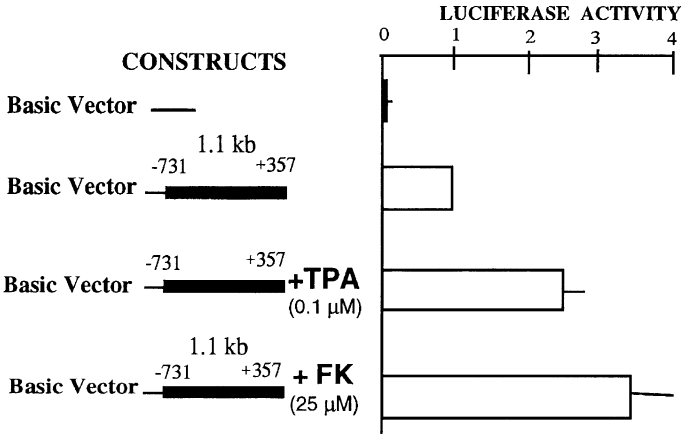

Fig. 7. Phorbol esters and cAMP activate *Kcn1* gene transcription. The 1.1-kb fragment was cloned in the promoterless basic plasmid and transfected in LLC-PK_1 cells. Cells were treated with TPA (n = 22) or forskolin (FK, n = 14). Transcriptional activity was measured by the luciferase assay. For a negative control, the Basic vector without insert was transfected. TPA and forskolin had no effect on the transcriptional activity of Basic vector alone (data not shown). Transcriptional activity of Basic vector with the 1.1-kb insert was normalized to 1, and values were reported as means ± SE.

we find that the glucocorticoid dexamethasone consistently inhibits *Kcn1* gene expression in LLC-PK_1 cells. Further studies are underway to fully examine this response. We recently isolated a second member of the Kcn family, Kcn2. The 5′-flanking region of the Kcn2 was sequenced. Although it is highly homologous to Kcn1, it contains several divergent areas in the enhancer region. The functional significance of these differences is being investigated.

In conclusion, we have identified a novel gene encoding a K^+-selective channel (Kcn1) that is expressed in vascular tissues and specifically activated by cGMP. It may play an important role in the regulation of arterial tone. We find that the coding region of Kcn1 is intronless. The promoter region was localized to a 193-bp region by reporter gene assay. A 5′ upstream enhancer element was detected and localized to a 150-bp segment. Phorbol esters (TPA) and forskolin significantly stimulated *Kcn1* gene expression. These studies provide a framework for a more detailed investigation of the regulation and the cell-specific expression of the *Kcn1* gene.

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