Genomic structure and promoter analysis of the rat Kir7.1 potassium channel gene (*Kcnj13*)

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Abstract In the brain inwardly rectifying potassium channel Kir7.1 subunits are predominantly expressed in the choroid plexus and meninges. To investigate this tissue-specific expression pattern, we characterized the genomic organization and the 5′ proximal promoter of the rat Kir7.1 gene (*Kcnj13*). Starting from the major transcriptional initiation site, three exons in *Kcnj13* give rise to the dominant ~1.45 kb transcript in brain. Adjacent to the transcriptional start the minimal promoter which, uncommon for ion channels, contains a TATA- and CAAT-box is controlled by AP-1 factors and accounts for high gene expression levels. Luciferase reporter gene responses driven by the first 2.1 kb of the 5′ flanking region were similarly high in epithelial FRTL-5 and neuronal N2A cells, suggesting that neuron-specific repressor elements are located remote from the non-selective minimal promoter.

Key words: Gene structure; TATA-box; CAAT-box; AP-1; Choroid plexus; FRTL-5

1. Introduction

Since the cloning of the first gene encoding a subunit of inwardly rectifying K^+ (Kir) channels in 1993, a total of 15 different subunits with conserved primary amino acid sequences but different functional characteristics have been identified in mammals [1]. Both in the developing and adult animal, they are expressed in a variety of tissues and have been found to control the resting potential, maintain K^+ homeostasis, transduce cellular metabolism into excitability and regulate cell growth and differentiation. The expression pattern of Kir transcripts in the brain is highly differential, with some members being ubiquitously expressed but others restricted to either neurons or glia, to brain structures segregated during ontogeny, or to nuclei associated with functional systems [2,3]. However, to date little is known about the molecular mechanism that controls the cell-specific expression of the different Kir genes.

The Kir channel subunit Kir7.1, which was recently isolated and mapped to chromosomal locus 2q37 in man (*Kcnj13*) [4], represents the most unusual member of this family: its primary structure is only moderately homologous to other subunits, and its functional properties are unique [5–7]. In particular, due to the presence of a non-conserved residue in the channel's outer pore region, Kir7.1 does not follow some of the salient features of ion permeation typical of classic Kir channels [8], e.g. it exhibits an extremely small unitary conductance of ~50 fS, and this conductivity is virtually independent of extracellular $[K^+]$. Of relevance in this research, in situ hybridization [6] and immunocytochemistry [9] revealed that in the brain Kir7.1 subunits are absent from neurons and glia but are likely expressed at unusually high density ($\leq 10^3$ channels/μm^2 cell membrane) within epithelial cells of the choroid plexus and meninges. Moreover, it was found that, in contrast to other transporting epithelia of the thyroid or intestine, Kir7.1 subunits are targeted to the apical side of choroid plexus cells, possibly in conjunction with a Na^+/K^+-ATPase [9]. So far no molecular signals that may be involved in the polarized targeting in epithelia have been recognized in Kir7.1. However, in other membrane proteins this segregation is likely controlled by sorting motifs in the primary structure of the COOH-terminus (basolateral) or transmembrane/extracellular region [10,11]. In contrast, tissue specificity and the level of expression are more likely determined by *cis*-acting elements located within the 5′ flanking regions of the gene [12,13]. To address the issue of the highly specific tissue expression of Kir7.1, we determined the genomic structure of the rat Kir7.1 (rKir7.1) gene, located the transcription initiation site and analyzed the promoter activity of the 5′ flanking sequence using a luciferase reporter assay in various cell lines.

2. Material and methods

2.1. Cell culture

Neuro2A neuroblastoma cells (N2A, No. CLL131, American Type Culture Collection, Rockville, MD, USA) and HEK293 cells (No. CRL1573) were maintained in Dulbecco's modified Eagle's medium, supplemented with non-essential amino acids (N2A), 10 mM HEPES (HEK293), 10% fetal bovine serum, 2 mM glutamine and 100 U/ml penicillin/streptomycin (HEK293 and N2A), at 37°C and 5% CO_2. FRTL-5 cells (No. 91030711; European Collection of Cell Cultures, Salisbury, UK), a strain of rat thyroid follicular cells, were grown in Coon's modified Ham's F12 medium supplemented with 5% fetal bovine serum, 2 mM glutamine, 10 μg/ml insulin, 10 nM hydrocortisol, 10 μg/ml transferrin, 10 ng/ml Gly-His-Lys acetate, 10 ng/ml somatostatin, 10 U/ml TSH and 100 U/ml penicillin/streptomycin at 37°C and 5% CO_2.

2.2. Protein extraction and Western blotting

For protein extraction N2A, HEK293 and FRTL-5 cells were washed with phosphate buffered saline (PBS) and harvested by scraping the cell layer and subsequent low speed centrifugation of the cell suspension. Cell pellets and tissue preparations from Wistar rats were homogenized in ice cold lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EGTA, protease inhibitors [1 μg/ml leupeptin and pepstatin A, 2 μg/ml aprotinin, 1 mM PMSF], 1% Triton X-100) and cleared from the non-soluble components by high speed centrifugation

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for 5 min at 4°C. Equal amounts of protein extracts (estimated using the BioRad protein assay) were separated by polyacrylamide gel electrophoresis and electroblotted onto Immobilon P membranes (Millipore, Badford, MA, USA). Western blots were probed with polyclonal rabbit Kir7.1 antibodies (1:1500; kindly provided by R. Veh, Berlin) and detected with horseradish peroxidase conjugated goat anti-rabbit immunoglobulins (1:5000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) using an enhanced chemiluminescence detection system (ECL; Amersham, Buckinghamshire, UK).

2.3. Genomic cloning

For analysis of the Kir7.1 gene structure, a rat genomic λ-phage library (DASH; Stratagene, La Jolla, CA, USA) was homology screened using a ^{32}P-labeled polymerase chain reaction (PCR) fragment comprising bp 642–1262 (Fig. 1) of the 3′ region of the rat Kir7.1 cDNA. Screening of a total of 10^6 plaques under high stringency conditions resulted in the isolation of six positive clones containing inserts between 12 and 15 kb in length. λ clones were plaque purified, and the DNA was purified using the Qiagen Lambda Midi Kit (Qiagen, Hilden, Germany) and sequenced from both sides with the BigDye terminator Kit on an ABI Prism 310 Genetic Analyzer (PE Applied Biosystems, Weiterstadt, Germany).

2.4. Reverse transcriptase and rapid amplification of cDNA ends (RACE)-PCR

Reverse transcription (RT)-PCR was carried out with Superscript II reverse transcriptase (Life Technologies, Gaithersburg, MD, USA) and 30 cycles of PCR amplification using Taq polymerase (Qiagen). 5′ RACE was performed according to the manufacturer's instructions (Life Technologies) with 350 ng poly(A)+ RNA from rat tissue (and FRTL-5 cells) as the template and a Kir7.1-specific primer (5′-TTAGGTTTGCCATCTTTGTGAGC-3′) located 520 bp downstream of the translational start site. In subsequent PCR amplifications, the first and nested primers were 5′-GTGAATCGGATTGAGAAAGC-3′ and 5′-CTGGGGAACATGGTGCCATAGC-3′ and were located 500 and 360 bp, respectively, downstream of the translational start site. PCR products were purified from agarose gels by QiaExII (Qiagen), and 60–90 ng of the DNA fragment were directly sequenced. For 3′ RACE, rat brain poly(A)+ RNA was reverse transcribed using a 3′ RACE adapter primer (Life Technologies). The subsequent PCR amplification was performed using a Kir7.1-specific primer (5′-GCAAGAAGGCACTGGGGAAATC-3′) located 340 bp upstream of the stop codon and an anchor primer homologous to the 3′ RACE adapter tail.

2.5. Luciferase reporter gene assay

The 5′ flanking sequences of the rKir7.1 gene were inserted into pGL3-Basic (Promega, Madison, WI, USA) to generate promoter/firefly luciferase constructs. The SV40 promoter in pGL3 was used as positive control. Promoter fragments of rKir7.1 were generated by PCR from the phage clone DNA, and fragment deletion of the parental reporter plasmid. Proofreading polymerases *pfu* (Stratagene) and *pfx* (Life Technologies) were used to minimize amplification errors, and constructs were confirmed by sequencing and restriction analysis. Plasmid DNA was purified by Qiagen anion exchange chromatography.

For analysis of reporter gene constructs, FRTL-5 cells were grown to confluency, washed with PBS, harvested using trypsin (0.1% in PBS/0.02% EDTA) and resuspended in a solution containing 120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4, 10 mM KH_2PO_4, 5 mM glutathione, 2 mM ATP, 2 mM EGTA, 5 mM $MgCl_2$ and 25 mM HEPES (pH 7.6). A 400 μl aliquot of cells ($\geq 1\times10^6$ cells) was electroporated (965 μF and 350 V) with 20 μg of the firefly luciferase reporter plasmid and 2 μg of the *Renilla* luciferase plasmid (pRL-TK or pRL-SV40; Promega) at a 10:1 ratio (Gene Pulser System, BioRad, Hercules, CA, USA) and subsequently replated. N2A and HEK293 cells, plated on 30 mm dishes, were transfected with 1 μg of firefly luciferase reporter plasmid and 0.1 μg *Renilla* luciferase plasmid. For manipulation of signaling pathways, 0.1 μg of the vectors carrying PKA and MAP/ERK kinase kinase (MEKK1; Stratagene) were cotransfected. Forty-eight h after transfection all cell lines were washed with PBS, exposed to 500 μl of 1× passive lysis buffer (Promega) for 15 min. Cytosolic cell extracts were cleared of debris by centrifugation. Twenty μl of the supernatant was assayed for luciferase activity on a Berthold luminometer using the dual luciferase reporter assay system (Promega).

3. Results and discussion

3.1. Genomic structure of Kcnj13

Homology screening of a rat genomic λ-phage library with a *Kcnj13* cDNA probe resulted in the identification of four overlapping clones, 4B, 6A, 5A2 and 10A, that cover a total of 24.6 kb of genomic DNA in length (Fig. 1A). Analysis of this sequence and comparison with the rKir7.1 cDNA [9] revealed that, typical of the multiple exon structure known

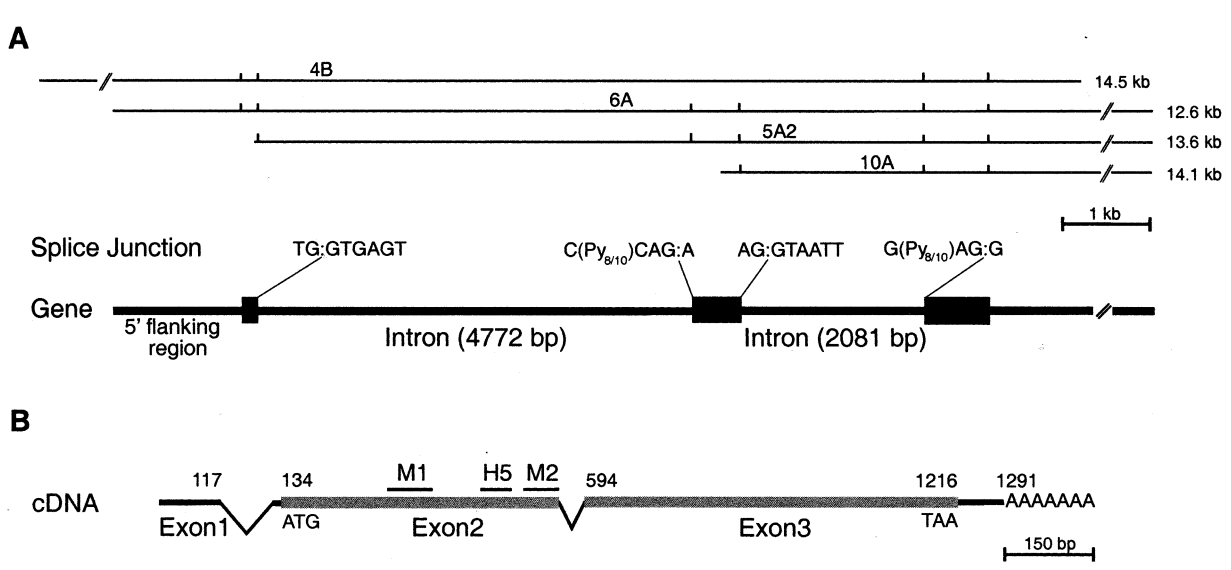

Fig. 1. Genomic structure of *Kcnj13*. A: Schematic representation illustrating that *Kcnj13* is composed of three exons (black boxes) and two introns flanked by conventional splice donor and acceptor sites. A total of 24.6 kb of genomic sequence have been deduced from the four overlapping genomic phage clones shown (4B, 6A, 5A2 and 10A). B: The positions of the transmembrane segments M1 and M2, and the pore region H5 are indicated in the rat brain Kir7.1 cDNA as determined from genomic and total RNA analysis. The ORF is shown as a shaded box, and 5′ and 3′ UTRs are represented by black lines. The gene bank accession number for the *Kcnj13* genomic sequence is AJ292748.

in Kir channels (e.g. [14–20]), the Kir7.1 cDNA is likely encoded by three exons. First, the open reading frame (ORF) of Kir7.1 is disrupted and split into two exons (exon 2 and 3) at a glycine (position 154) in the COOH-terminal part of transmembrane segment M2 by an intron which is 2081 bp in length and flanked by conserved splice 5′ donor and 3′ acceptor sites (Fig. 1). In exon 3, only 31 bp downstream of the stop codon, a putative polyadenylation signal sequence (5′-ATTAAATATA-3′) was identified. 3′ RACE-PCR with whole rat brain RNA revealed that in Kir7.1 transcripts the poly(A) tail is indeed adjoined to the polyadenylation site at a distance of 33 bp, yielding a total length of 698 bp for exon 3.

To determine the 5′ structure of *Kcnj13* and the exact transcriptional initiation site we performed 5′ RACE-PCR with whole rat brain RNA. Using antisense primers derived from the rKir7.1 ORF together with 5′ RACE-specific primers a single PCR product was amplified, indicating one major transcriptional start site for Kir7.1 mRNAs in the rat brain. Sequence analysis of the 5′ UTR uncovered 133 bp upstream of the start codon, in which the initial 117 bp are coded by exon 1 and the following 16 bp by exon 2 (476 bp). Further sequence analysis revealed that the intron separating both exons is 4772 bp in length (Fig. 1A). Consequently, the Kir7.1 cDNA in rat brain analyzed here comprises 1291 bp plus a poly(A)+ tail (Fig. 1B) which closely agrees with the major ~1.45 kb transcript detected in Northern blots of rat brain RNA [6].

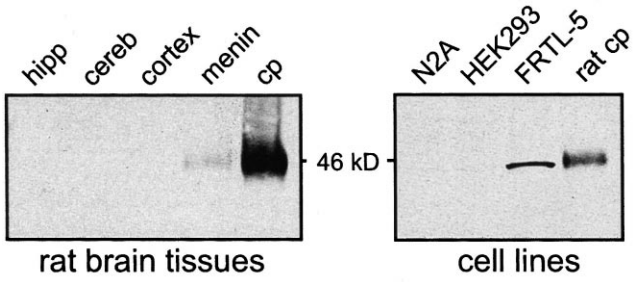

Fig. 3. Western blot analysis of Kir7.1 in rat brain and cell lines. Left panel: 20 μg total protein from rat hippocampus (hipp), cerebellum (cereb), cortex, meninges (menin) and choroid plexus (cp) were probed with polyclonal rabbit Kir7.1 antibody (1:1500). Right panel: total cell extracts of N2A, HEK293 and FRTL-5 cells (20 μg) and rat cp (3.5 μg) serving as control were analyzed for Kir7.1 expression. No signals were detected in parallel blots of FRTL-5 cells and cp that were probed with Kir7.1 antibodies preincubated with the antigen (data not shown).

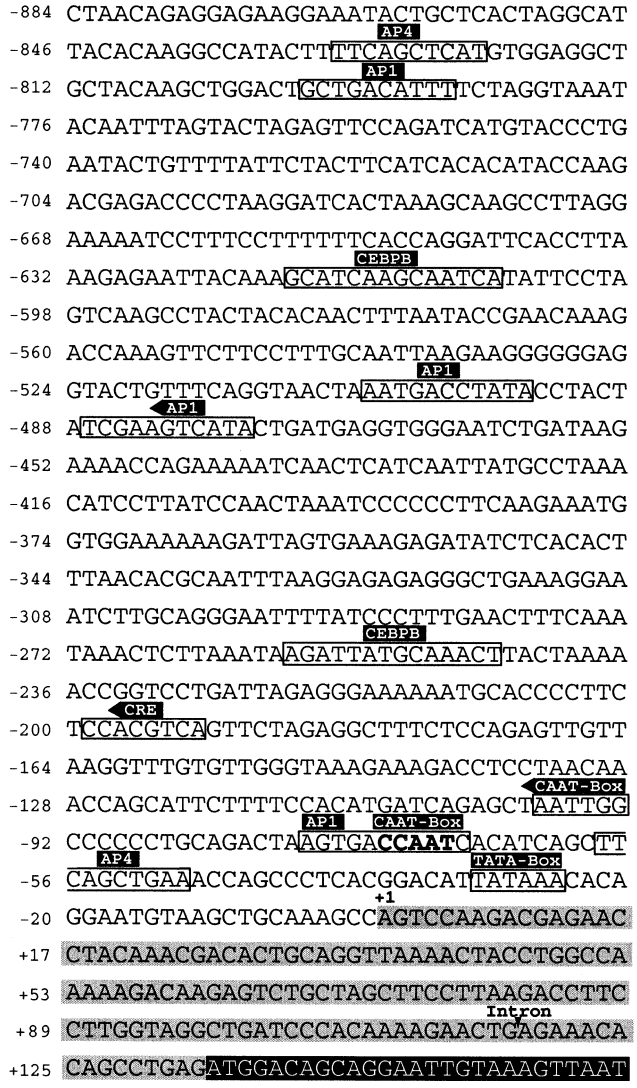

Fig. 2. 5′ region of the rat Kir7.1 gene. Nucleotide sequences of the 5′ flanking region (884 bp) of *Kcnj13* and the initial part of cDNA are shown. Numbers represent nucleotide positions relative to the transcriptional initiation site (+1). The 5′ UTR of the cDNA and the partial ORF are shaded in gray and black, respectively. Potential transcription factor binding sites are depicted. Inverted consensus sequences are marked by arrowheads.

3.2. Structural analysis of the Kcnj13 5′ flanking region

Approximately 800 bp of the 5′ flanking region upstream of exon 1 in the λ-phage clone 6A were sequenced as an initial step to analyze the *Kcnj13* promoter (Fig. 2). A consensus TATA-box (TATAAA) at position −30 and a CAAT-box at position −72, which together mediate transcriptional initiation via TFIID and RNA polymerase II [21–23], were immediately detected in the core promoter in close proximity to the initiator region. Computer analysis with the MatInspector program [24] identified further *cis* regulatory elements in the rat gene: four AP1 sites (positions −77, −474, −505 and −797), known to be targets for c-Jun/c-Fos heterodimers (one of them overlapping with the CAAT-box); an inverted CAAT-box (position −93); two recognition motifs for helix–loop–helix AP4 transcription factors (position −58, −831); two CAAT-box enhancer binding protein β sites, known to regulate cellular differentiation [25]; and an inverted CRE sequence (position −207), recognized by stimulus-induced transcription factors of the CREB family [26]. A BLAST search [27] of the recently released sequence database of the Human Genome Project with the rKir7.1 genomic sequence identified the 5′ flanking region of the hKir7.1 gene (accession number AC016692) which shows 90% identity with the initial 422 bp of the rKir7.1 5′ flanking region including all *cis* regulatory elements.

3.3. Promoter activity of the Kcnj13 5′ flanking region

In situ hybridizations in rat brain identified Kir7.1 transcripts only in epithelial cells of the choroid plexus and the meninges but not in neurons [6]. These results are confirmed

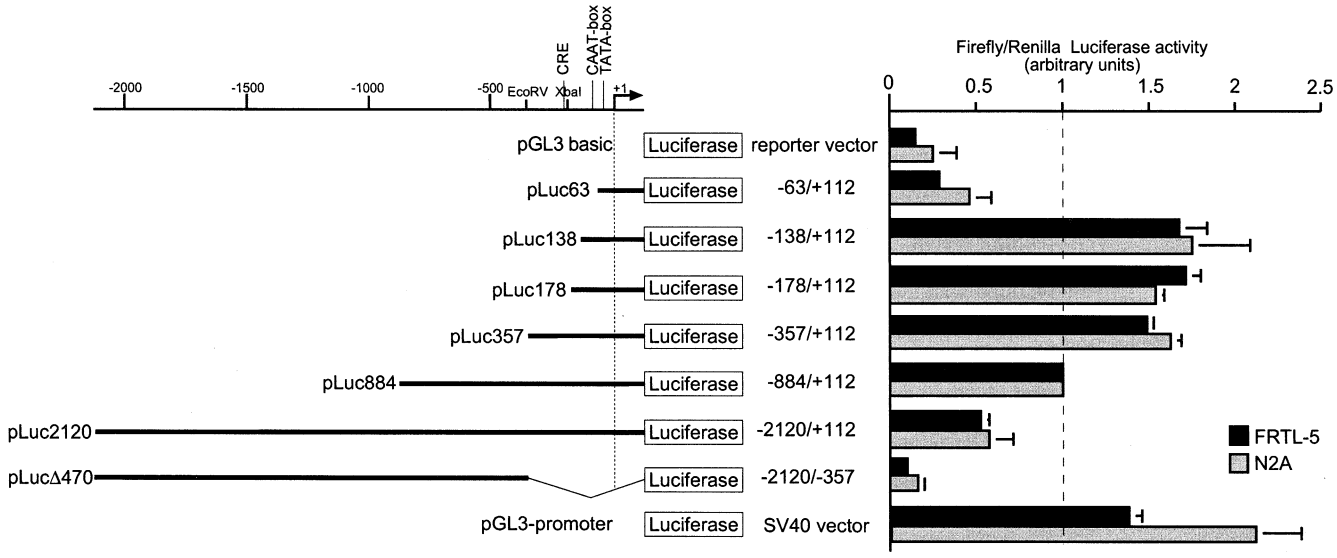

Fig. 4. Analysis of Kir7.1 promoter activity in FRTL-5 and N2A cells. FRTL-5 (black bars) and N2A (gray bars) cells were transfected with each of the pLuc plasmids containing the firefly luciferase reporter under control of different fragments of the Kir7.1 promoter. Total luciferase activity was measured after 48 h. The efficiency of transfection was normalized by co-transfecting the *Renilla* luciferase vector pRL-TK, and promoter activity was normalized to the activity of the pLuc884 construct (broken line). Results are presented as mean ± S.D. ($n = 3$). Position of the transcriptional initiation site (+1) is indicated by the dotted line.

here on the protein level by Western blots in which equal amounts of whole cell protein from different brain tissues were blotted. Fig. 3 (left panel) shows several-fold higher levels of Kir7.1 protein in choroid plexus compared to the meninges and the absence of Kir7.1 from neuronal cells. To assess a potential role of promoter elements in the 5′ flanking region of *Kcnj13* on this differential distribution, we investigated their ability to drive expression of the luciferase reporter gene in two representative cell lines: FRTL-5 cells originating from thyroid gland and N2A neuroblastoma cells. We found that FRTL-5 cells use the same transcriptional start site at position −133 as determined for the rat brain (data not shown), and Nakamura et al. [9] have recently reported on the expression of Kir7.1 mRNA in this cell line. Fig. 3 (right panel) documents that Kir7.1 protein is strongly present in FRTL-5 but absent from both N2A and HEK293 cells (also confirmed by RT-PCR), and thus demonstrates the suitability of these cell lines for a comparative promoter analysis. Initially, 996 bp, consisting of 884 bp 5′ flanking region and 112 bp of exon 1, were inserted into the pGL3 basic vector (termed pLuc884), and extracts from each transfected cell line were assayed for luciferase activity. pGL3 basic which represents the low background of the reporter vector, and pGL3-promoter, in which luciferase expression is driven by the SV40 promoter, were used as controls. To identify discrete regions controlling transcriptional activity, several deletion constructs of pLuc884 as well as a construct with a 2120 bp 5′ flanking region identified from λ-phage clone 4B (pLuc2120) were tested for their reporter gene response (Fig. 4).

Our data show that, in general, the relative promoter activity of all *Kcnj13* constructs obtained in N2A cells did not differ significantly from that obtained in FRTL-5 thyroid gland cells. These findings indicate that regulatory elements mediating cell-specific expression are absent at least 2120 bp upstream of the transcription site. In more detail, with pLuc884 used as standard, the luciferase activity in constructs generated by successive deletions of pLuc884 (pLuc357, pLuc178, pLuc138) was enhanced by 50–70% in both cell lines. Of all analyzed constructs, pLuc138 showed the highest transcriptional activity which was comparable to the activity driven by the viral SV40 promoter in pGL3-promoter. However, further deletions of another 75 bp (pLuc63) which removed the CAAT-box (−73) resulted in a 60–70% decrease of luciferase activity relative to pLuc884. This dramatic reduction of luciferase activity indicates that pLuc138 comprises the minimal promoter of *Kcnj13*. The essential function of this region is also demonstrated in pLucΔ470 that lacks 470 bp upstream of the transcription initiation site and showed a luciferase activity comparable to the pGL3-basic control (Fig. 4).

An elongated 5′ flanking region beyond bp 884 as in the reporter plasmid pLuc2120 revealed transcriptional activity

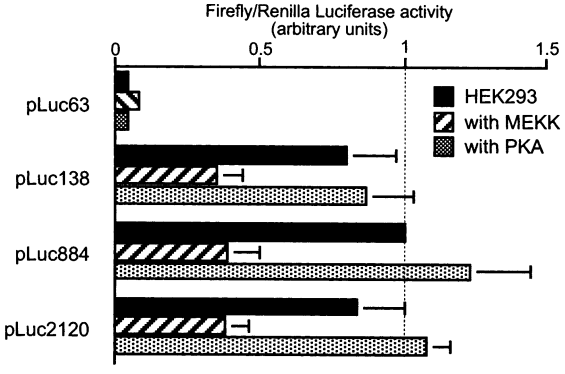

Fig. 5. Influence of signaling pathways on the activity of the *Kcnj13* promoter. HEK293 cells were individually transfected with various pLuc luciferase reporter constructs (black bars) or co-transfected with both constitutively active MEKK (hatched bars) and catalytic PKA subunits (dotted bars). Luciferase activity was measured and normalized to pLuc884 as described in the legend to Fig. 4.

that was reduced by 40% compared to pLuc884, but again almost identical for both cell lines and, thus, appears to lack cell-specific regulatory elements. In summary, we conclude that, independent of the host cell line, most of the luciferase activity of the analyzed constructs depends on strong core promoter elements in Kir7.1 that mediate an effective binding of RNA polymerase II.

In search for *cis* regulatory elements that could affect transcriptional efficiency in response to cellular signaling pathways, we first investigated a CRE site (position −201) originally identified as a target for phosphorylated CREB and thus being involved in cell proliferation, differentiation and adaptive processes [26]. Because CRE binding protein transcription factors bind to CRE after PKA phosphorylation at amino acid position 133 [28], catalytic subunits of PKA were transfected together with the various luciferase reporter constructs in HEK293 cells. The results shown in Fig. 5, however, demonstrate that overexpressed PKA subunits only insignificantly enhanced the luciferase activity of all tested constructs, indicating a negligible role of the CRE site in the *Kcnj13* promoter.

In contrast, the AP1 site that overlaps with the CAAT-box in the core promoter (position −72) was found to negatively affect the transcriptional activity of the Kir7.1 promoter. AP1 sites bind c-Jun/c-Fos heterodimeric transcription factors following phosphorylation and activation by c-Jun N-terminal kinase, a MAP kinase isoform [29], which in turn is activated by MEKK [30]. We therefore investigated the function of the AP1 site by co-transfecting reporter constructs with an expression vector encoding constitutively active MEKK (Stratagene). Fig. 5 shows that constructs that comprise the CAAT-box/AP1 region yielded luciferase activity levels that were generally reduced by ∼50%, whereas in pLuc63 in which the CAAT-box/AP1 site was deleted, the activity rather increased in the presence of MEKK. Thus, as has been suggested for NF-Y and SP1 transcription factors in the mouse Kir2.1 gene [18], binding of activated c-Jun/c-Fos close to the transcriptional initiation site of *Kcnj13* may negatively interfere with binding of the basal transcriptional apparatus under our experimental conditions.

In summary, we have shown that in epithelial Kir7.1 subunits high transcriptional levels that compare to those driven by the SV40 promoter are dependent on the presence of strong core promoter elements, a TATA-box and a CAAT-box, adjacent to the transcriptional start site. Similarly, these motifs are present in the 5′ flanking region of epithelial Kir1.1 channels [15] but are absent from the promoters of Kir subunits typically expressed in the central nervous system (e.g. [16,18,31]). In the genes of K^+ channel subunits analyzed so far the activity of the minimal promoter is strongly controlled in a tissue-specific manner by *cis*-acting elements. In both Kv1.5 and Kir2.1 subunits, repressor/silencer elements that differentiate between neuronal and non-neuronal cells have been identified close to the transcriptional start site [13,18].

Generally, tissue-selective repressor or enhancer elements in various genes have been located within intronic or exonic sequences in the proximal part of the 5′ flanking region (e.g. REST; [32–34]) as well as several ten thousand bases up- or downstream of the coding region [35,36]. Our reporter gene analysis indicates that within the first 2120 bp upstream of the transcriptional start site in *Kcnj13*, despite some general repressor activity, no tissue-specific regulatory elements are present. In the future bacterial artificial chromosomes (> 100 kb) containing the Kir7.1 gene might represent a useful extension to our study to search for remote regulatory elements that confer neuron-specific repression or transcriptional enhancement in epithelia.

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