Cloning of a New Mouse Two-P Domain Channel Subunit and a Human Homologue with a Unique Pore Structure*

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Mouse KCNK6 is a new subunit belonging to the TWIK channel family. This 335-amino acid polypeptide has four transmembrane segments, two pore-forming domains, and a Ca2+-binding EF-hand motif. Expression of KCNK6 transcripts is principally observed in eyes, lung, stomach and embryo. In the eyes, immunohistochemistry reveals protein expression only in some of the retina neurons. Although KCNK6 is able to dimerize as other functional two-P domain K+ channels when it is expressed in COS-7 cells, it remains in the endoplasmic reticulum and is unable to generate ionic channel activity. Deletions, mutations, and chimera constructions suggest that KCNK6 is not an intracellular channel but rather a subunit that needs to associate with a partner, which remains to be discovered, in order to reach the plasma membrane. A closely related human KCNK7-A subunit has been cloned. KCNK7 displays an intriguing GLE sequence in its filter region instead of the G(Y/F/L)G sequence, which is considered to be the K+ channel signature. This subunit is alternatively spliced and gives rise to the shorter forms KCNK7-B and -C. None of the KCNK7 structures can generate channel activity by itself. The KCNK7 gene is situated on chromosome 11, in the q13 region, where several candidate diseases have been identified.

Ion channels are present in excitable and nonexcitable eukaryotic cells, where they control the electrical potential across the cell membrane, secretion, and signal transduction (1). Electrophysiological studies have allowed identification and characterization of a great variety of ion channels, which are differentiated first by their selectivity and then by their other biophysical properties, their pharmacology, and their regulation (2). Recent cloning efforts and analysis of structure-function relationships have now provided a molecular basis for many of the biophysical properties. Thus, the mechanism that determines ion channel selectivity is now fairly well understood for K+ channels (3). K+ selectivity is carried by a structural element called P domain (P for pore-forming). This domain is highly conserved among the three main structural classes of K+ channel subunits with six-transmembrane, four-transmembrane, or two-transmembrane domains (4).

The most recently described family of K+ channels has four transmembrane domains (5–10). Members of this family have two pore-forming domains (P1 and P2), an extended extracellular loop between the M1 and P1 domains with a conserved cysteine residue (except for TASK-1), and a short cytoplasmic N-terminal extremity. TWIK-1 has been shown to form covalent homodimers (8, 11). Despite a relatively low sequence similarity and different functional properties, these two-P domain K+ channels all produce quasi-instantaneous and noninactivating currents, although the TASK-2 currents display relatively slow activation kinetics. These new K+ channels are presently classified into three distinct functional subfamilies. TASK-1 and TASK-2 are background K+ channels sensitive to small external pH variations near physiological pH (9, 10, 12). TREK-1 (13) and TRAAK (6, 14) are arachidonic acid-activated mechanosensitive K+ channels; however, TREK-1 is inhibited by both protein kinase A and protein kinase C (5), while TRAAK is not. TWIK-1 is a weakly inward rectifying K+ channel that is stimulated by PKC and inhibited by internal acidification (7, 8). The different types of regulations indicate that these K+ channels are probably involved in a great diversity of physiopathological and pathophysiological roles.

More than 40 genes potentially encoding two-P domain K+ channel subunits have now been identified in the Caenorhabditis elegans genome (15). This observation suggests that a large number of two-P domain channels might also exist in mammals. The present paper describes the cloning of two novel mammalian two-P domain channels related to TWIK-1, one of mouse and the other of human origin.

EXPERIMENTAL PROCEDURES

Cloning of KCNK6 and KCNK7—A BLAST search using the TWIK K+ channel led to the identification of mouse and human expressed sequence tags (ESTs); accession numbers W18545 and AA777882. In order to characterize the corresponding full-length cDNAs, called KCNK6 and KCNK7, 5′- and 3′-rapid amplification of cDNA ends (RACE) were performed on adult mouse or human brain cDNAs ligated with adapters as previously (16). Two antisense primers for 5′-RACE (5′-CTCACCAGGGCCTCAGGGC-3′) specific to KCNK6 and 5′-AGGCCCTTCTCCCGCCTGGGAT-3′ specific to KCNK7 and two sense primers for 3′-RACE (5′-TGGAGGCACAGGGACCATGGAGT-3′) specific to KCNK6 and 5′-TGCCCTGCTGACGAGCATTT-3′ specific to KCNK7) were derived from ESTs W18545 and AA777882. Two successive RACE reactions were performed with a mixture of Taq (Life Technologies, Inc.) and Pwo (Boehringer Mannheim) DNA polymerase. A Luminon Gold signal was captured in an agarose gel, and the amplified fragments were sequenced.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AF110521 (KCNK6), AF110522 (KCNK7-A), AF110523 (KCNK7-B), and AF110524 (KCNK7-C). To whom correspondence should be addressed: Institut de Pharmacologie Moléculaire et Cellulaire, CNRS, 660 route des Luccioles, Sophia Antipolis, 06560 Valbonne, France. Tel.: 33 4 93 95 77 02 or 33 4 93 95 77 03; Fax: 33 4 93 95 77 04; E-mail: ipmc@ipmc.cnrs.fr.

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The abbreviations used are: EST, expressed sequence tag; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; GST, glutathione S-transferase; ER, endoplasmic reticulum.
polymerases by using anchor primers 5'-TAGAATGGGTGCTGACGCAGG-TATC-3' and 5'-GATTAGTTGGTACATAGATAATCG-3', and three clones of each protein were sequenced on both strands (Applied Biosystems model 373A). Primers flanking the variable region (primer F, 5'-CTGCTACTCTTCTCTGCTGTC-3'; primer R, 5'-TACGACAGGAGGCGGGTTG-3') were used to amplify the different splicing forms from human brain cDNA (10 ng) with the low error rate DNA polymerase (Pwo, Boehringer Mannheim). The PCR products were digested with EcoRI and BamHI and subcloned into the pIRE5-DS expression vector (6) and sequenced. In the same way, the coding sequences of both splicing forms KCNK7-B and KCNK7-C were amplified with the KCNK7-A sense primer and the antisense primers: KCNK7-B, 5'-CCGCGATCCC-GGCTACCCGCGGT-3' and KCNK7-C, 5'-CCGCGATCTCCGATGACGGTTGTTCTC-3'.

**Analysis of KCNK7 Exon Skipping**—Primers flanking the variable region (primer F, 5'-CTGCTACTCTTCTCTGCTGTC-3'; primer R, 5'-TACGACAGGAGGCGGGTTG-3') were used to amplify the different splicing variants from rabbit brain cDNA (10 ng) with the low error rate DNA polymerase (Pwo). Fragments were separated by agarose gel (2.5%) electrophoresis, and fragments were analyzed on an agarose gel. Southern blot analysis was performed with the blot analysis, mouse and human multiple-tissue Northern blots (CLONTECH). The cDNA probes were labeled with [32P]dCTP (Amersham Pharmacia Biotech). Blots were saturated with 4% low fat dry milk in PBS and incubated 1 h with affinity-purified anti-KCNK6 antibodies or M2 anti-tag monoclonal antibodies (Eastman Kodak Co.) diluted 500-fold, followed by an additional 1-h incubation with horse- or rabbit-anti-mouse IgG (Jackson) with extensive washing steps after antibody incubation, and finally revealed with Super Signal (Pierce).

**Indirect Immunofluorescence on Transfected COS-7 Cells**—Transfected COS-7 cells were plated on glass coverslips onto 15-mm Petri dishes. 24 h after plating, the cells were fixed for 15 min with 4% (v/v) paraformaldehyde/PBS solution, rinsed with PBS, and then permeabilized by incubation for 10 min with 0.1% Triton X-100 (without Triton for nonpermeabilized conditions). Nonspecific binding was eliminated by a 2-h incubation with 5% goat serum, 2% bovine serum albumin in PBS at room temperature. The cells were then incubated for 1 h with affinity-purified anti-KCNK6 antibody (1:400) or anti-TRAAK antibody (1:400)2 in 2% bovine serum albumin/PBS, followed by washing with PBS containing 2% goat serum to remove unbound antibody. The fixed and serum-depleted cells (see below for the transfection) were incubated with a 1:100 dilution of affinity-purified anti-KCNK6 antibody (1:400) or anti-TRAAK antibody (1:400), washed with PBS, and then fixed with cold 4% (v/v) paraformaldehyde/PBS for 2 h. The latter conditions were used to allow sufficient fixation of the target proteins to permit efficient antibody incubation. Between each antibody incubation, the cell surface was stained with a 1:200 dilution of goat-anti-mouse IgF (Jackson) with extensive washing steps after antibody incubation, and finally revealed with Super Signal (Pierce).

**Construction of N-terminally Tagged KCNK6 and KCNK6 Mutants**—The coding sequence of KCNK6 was subcloned into the HpaI–NotI restriction sites of Flag-pRc/CMV vector to create an N-terminally tagged KCNK6 protein as described previously for the N-terminally tagged Kv2.2 protein (19). Deletion chimeras and chimeras between KCNK6, mTREK1, and hTASK1 were constructed by PCR as described (20). PCR was performed on Flag-pRc/CMV/KCNK6 to keep the Flag peptide sequence at the amino extremity of the constructions. Products were subcloned into EcoRI–BamHI site of pIRE5-DS vector and verified by sequencing.

**Cell Culture and Transfection—COS-7 cells were grown in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum and antibiotics (10 mg/ml penicillin, 50 mg/ml streptomycin). One day before transection, 20 x 10^6 cells for electrophysiological experiments or 70 x 10^6 cells for immunofluorescence microscopy were plated on cover glasses onto 35-mm Petri dishes. The cells were transfected by the classical DEAE-dextran/chloroquine method using 1 or 2 μg of supercoiled DNA.

**Human Chromosomal Mapping of KCNK7—**Gene mapping was performed by PCR on the Genebridge 4 radiation mapping panel (Research Genetics) with the following primers: sense, 5'-CCGCGATCTCAGCAGCAGGTTGTTCTC-3'; antisense, 5'-CTGGACGTGCAGGACCCAGG-3'. The results were analyzed by using the RHMAPPF program at the Whitehead Institute with a logarithm of odds score of 21.

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Amino Acid Sequence Alignments and Dendrogram—The alignment was generated with the ClustalW multiple sequence alignment program. The dendrogram and the percentage of identity were deduced from the conserved region extending from the M1 to the M4 transmembrane domains. Accession numbers of the channel aligned are as follows: mTWIK, AF033017; mTRA9K, AF056492; hTASK-1, AF006823; hTASK-2, AF084830; mTREK1, U73488.

RESULTS

Cloning of KCNK6, a New Ion Channel Subunit—The TWIK-1 sequence (7) was used to search related sequences in the Genbank data base by using the Blast alignment program. A mouse EST (accession number W18545) was identified encoding a portion of a new α-subunit (named KCNK6). The corresponding full-length cDNA was obtained by RACE and sequenced. The coding sequence was then reamplified from both mouse brain and lung cDNAs to verify the sequence and to exclude all possible PCR errors. The KCNK6 cDNA contains an open reading frame of 1008 base pairs and codes for a protein of 335 amino acids (Fig. 1A) with a calculated molecular mass of 35 kDa. KCNK6 is more related to TWIK-1 than to the other two-P domain K+ channels (40% of amino acid identity (Fig. 1C)). Like all of these channels, KCNK6 displays four potential transmembrane segments (M1–M4) and two P domains (P1 and P2, P for pore-forming domain) (Fig. 1, A and B). Another important characteristic is the presence of an extended extracellular loop between M1 and P1 that contains a cysteine residue at a position (Cys57) analogous to the cysteine residue (Cys60) involved in the disulfide-bridged homodimerization of TWIK-1 (11). The protein sequence of KCNK6 contains consensus sites for N-linked glycosylation (residue 82) and phosphorylation by protein kinase C (residue 3) and casein kinase II (residues 255, 274, 276, 308, and 327). Three potential Src homology 3 binding motifs are present in the C-terminal part of the protein (minimal consensus sequence PXXP (21, 22)). Computer-based analysis also indicates the very interesting presence of a potential Ca2+-binding site (EF-hand motif) at its C terminus (Fig. 1D). This region includes the complete EF-hand loop as well as the first residue that follows the loop, which is always hydrophobic. Moreover, as in a classical EF-hand domain (23–25), the 12 residues loop of this region of KCNK6 are flanked on both sides by α-helical structures (nnPredict program; data not shown).

Tissue Distribution of KCNK6—In order to investigate KCNK6 mRNA expression, a Northern blot analysis was carried out using a mouse multiple-tissue Northern blot from CLONTECH. No KCNK6 transcripts were detected, indicating that KCNK6 is not expressed or is only expressed at very low levels in the tested tissues (heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis). The expression of KCNK6 was further studied on a larger panel of tissues with a more sensitive technique, reverse transcription-PCR (Fig. 2A). KCNK6 mRNA was detected in embryo (16–18 days), in eye, stomach, and lung. A weak expression was found in colon and testis. In the other tissues, the expression of KCNK6 mRNA was very low (atria, kidney, intestine, bladder, uterus, ovary, salivary gland, thymus, and brain stem) or not detectable (brain, cerebellum, spinal cord, heart, ventricle, skeletal muscle, liver, placenta, pancreas). In situ hybridization was performed on a vertical section of mouse retina. An antisense KCNK6 cRNA probe revealed an intense expression with a distinct stratification pattern. High labeling was apparent in the retinal ganglion cell layer (RGC) and in the inner nuclear layer (INL) composed of nuclei of Müller cells and amacrine, bipolar, and horizontal neurons (Fig. 2B). The distribution of the hybridization signal suggests a neuronal localization of transcripts. The control sense probe did not show significant hybridization (data not shown). To confirm these results, immunohistochemistry was performed on an equivalent section. The KCNK6 protein was immunodetected in the same neurons. A strong immunoreactivity was observed in the retinal ganglion cell layer. In addition, regular punctual staining was detected in a specific neuron population of the distal area of the inner nuclear layer (Fig. 2C). To demonstrate the specificity of anti-KCNK6 antibodies, an equivalent section was incubated with anti-KCNK6 antisera, which was previously absorbed with the GST-KCNK6 fusion proteins. As expected, the staining was prevented (data not shown). In situ hybridization and immunohistochemistry experiments did not show significant detection in the brain (data not shown).

Heterologous Expression of KCNK6 Subunits—Electrophysiological attempts to record KCNK6 channel activity in various cell types like Xenopus oocytes, COS-7, HEK293, and Sf9 cells were unsuccessful. In all of these expression systems, attempts to activate channel activity with 1 μM Ca2+ at the intracellular side (because of the presence of the EF-hand domain), with external pH variations from pH 7.0 to 4.0, or with application of a phorbol ester (phorbol 12-myristate 13-acetate) (there is one potential protein kinase C site (Fig. 1A)) were without any success. We also tried a coexpression with the β1γ subunits of G proteins, which lead to activation of G-protein-gated inward rectifier (26), as well as coexpression with the closely related two-P domain subunit TWIK-1, but again without success. Constructions with pCI and pRc/CMV expression vectors were also tested with the same negative results.

Western blot analysis was then carried out to verify KCNK6 expression in transfected cells. Under nonreducing conditions, anti-tag antibodies (M2) detected a major band at a molecular mass of 83 kDa from COS-7 cells transfected with a tagged form of KCNK6. Under reducing conditions (i.e., in the presence of β-mercaptoethanol), two bands were detected with molecular mass of 44 and 40 kDa. A similar blot profile was revealed using affinity-purified anti-KCNK6 antibodies (Fig. 3B). In order to demonstrate the specificity of the anti-KCNK6 antiserum and to justify its use to analyze the expression of the KCNK6 protein in transfected cells, we checked that no signal was obtained from control COS-7 cells expressing the TWIK-1 protein. This expression of the human TWIK-1 protein was verified in the same experiment with the affinity-purified rabbit antibodies directed against the C-terminal part of TWIK-1 (11) (data not shown). The masses of 37 and 40 kDa are in good agreement with the theoretical values of 35 and 37 kDa for KCNK6 and its tagged form, respectively. As for the TWIK-1 subunit (11), the observation that the band moving at 77 kDa gives rise to a band moving at 37 kDa in the presence of a reducing agent strongly suggests that KCNK6 can self-associate via a disulfide bond to form a homodimer. The masses of 41 and 44 kDa for KCNK6 and its tagged form suggest, as previously demonstrated for TWIK-1 (11), the existence of glycosylated forms. All of these results were reproduced in Sf9 cells (data not shown).

Intracellular Retention of KCNK6—One possible reason for the lack of channel activity could be that of being an intracellular localization of the expressed KCNK6 protein. To test this point, the KCNK6 subunit was localized by indirect immunofluorescence with the anti-KCNK6 antibodies (Fig. 3C) on Triton-permeabilized COS-7 cells. A strong fluorescence staining was detected at the perinuclear region as well as in a fine reticular network extending through the cytoplasm. This pattern was observed in all of the KCNK6-expressing cells from multiple independent transfections. This pattern is similar to that seen with known ER markers (27), suggesting that KCNK6 is specifically sequestered in the ER and cannot reach the plasma membrane. This pattern of labeling is very different from that
FIG. 1. A, alignment of the mouse amino acid sequences of KCNK6 (accession number AF110521) with mouse TWIK, TREK, TRAAK, and human TASK-1. The four transmembrane segments, M1–M4, and the two pore-forming regions P1 and P2 are underlined. Potential cytoplasmic sites for protein kinase C (●) and casein kinase II (Œ) are shown. On the cytoplasmic C terminus, potential Src homology 3 binding motif (PxxP) and EF-hand Ca\(^{2+}\)-binding domain are underlined. The conserved N-glycosylation site (Y) on the extracellular loop M1-P1 are shown. Identical residues are enclosed in solid boxes, while conservatively related residues are in shaded boxes. B, topological model for KCNK6 deduces from hydropathy analysis. As in A, the potential sites and putative domains are indicated on topological model. C, proposed dendrogram for the extended two-P domain K\(^+\) channel family. The percentage indicates the identity between the α-subunits. D, details of the consensus pattern of EF-hand Ca\(^{2+}\)-binding domain (identified with the Prosite program) and alignment of this EF-hand motif of KCNK6 with the EF-hands from calmodulin from Pneumocystis carinii (CaM), with L-type Ca\(^{2+}\) channel (L-Type CaC), and with the two EF-hands of outward rectifying two-P domain K\(^+\) channel from Arabidopsis thaliana (KCO1-EF1 and KCO1-EF2). The residues denoted by X, Y, Z, −Y, −X, and −Z are involved in coordinating Ca\(^{2+}\). X, any amino acid; braces, excluded amino acid; brackets, conserved amino acid.
obtained with the TRAAK channel. As shown in Fig. 3D, TRAAK-expressing cells, which produce strong K⁺ currents (6), show a typical surface labeling.

Why Is KCNK6 Unable to Reach the Plasma Membrane?—
The first reason why KCNK6 cannot reach the surface membrane could reside in the peptide sequence itself. Protein retention in intracellular compartments is usually determined either by the presence of short transmembrane domains (27–30) or by the presence of specific cytoplasmic sequences. These are the ER retrieval signals (KDEL, KK, or RR (31)), the peptide signals targeting to the trans-Golgi network (SXYQRL (33), AYRV (34), or SDSEED (35, 36)), the signals involved in endosomal trafficking (YKGL (35, 36)), or the endocytosis signal (DAKTI or DAKSS (37)). The transmembrane domains of the KCNK6 protein present the requisite length to reach the plasma membrane as for the other functional two-P domain K⁺ channels. Then a possible retention signal was searched on the cytoplasmic regions of the KCNK6 subunit. A particular sequence was found in the cytoplasmic tail between Ser²⁷⁴ and Asp²⁹⁶. This sequence SCKIIDSCKIEEDE is similar to a signal required for trans-Golgi network localization, SCKIKDSCKIKEDDEE (CKII indicates amino acids phosphorylated by casein kinase II) (35). This specific localization requires a cluster of acidic amino acids and a pair of amino acids phosphorylated by casein kinase II (38). Interestingly, this KCNK6 region overlaps with the EF-hand domain and in particular the residues that are expected to be involved in the coordination of Ca²⁺. One possibility would then be that the function of the putative retention signal could be eliminated by Ca²⁺ fixation. To test this point, an ionomycin/Ca²⁺ (1 μM) treatment of 5, 10, or 15 min at 37 °C was performed on transfected COS-7 cells, as described previously (39). Unfortunately, indirect immunofluorescence microscopy with anti-KCNK6 antibodies did not show any plasma membrane staining on cells having undergone this treatment (not shown). In addition, suppression of this potential retention signal in the KCNK6ΔC274/296 deletion mutant (Fig. 4) also failed to create access of KCNK6 to the plasma membrane. The immunolocalization of this mutant displays a pattern identical to that of the wild type subunit, and no current could be recorded in electrophysiological experiments. Other deletion mutants (KCNK6ΔC296, KCNK6ΔC282, KCNK6ΔC273) corresponding to the C terminus domain, where retention signals are generally localized, were constructed and expressed (Fig. 4). Again, the intracellular localization of these mutants was the same as for native KCNK6, and these mutants were electrophysiologically inactive. In order to suppress all possible retention signals on the KCNK6 cytoplasmic parts, chimeras have been prepared between KCNK6 and intracellular parts of the two-P domain TASK1 or TREK1 channels, which by themselves reach the plasma membrane (5, 9). The KCNK6/TASK-C and KCNK6/TREK-loop chimeras presented in Fig. 4 were produced and expressed. Again, the same characteristic ER lacy pattern was observed by immunocytolocalization of these chimeras, and no current was recorded. Taken together, these results show that the KCNK6 subunit remains inside of the cell but that it is probably not specifically expressed in the ER, since this subunit does not contain specific retention signals. The results suggest that KCNK6 necessitates an association with a partner to reach the plasma membrane. This possible partner seems to be absent in the expression systems used.

Cloning of KCNK7, a Human Subunit Closely Related to KCNK6—Since KCNK6 was cloned from mice and did not produce a functional channel, we then decided to clone a homologue of KCNK6 in humans in order to verify whether changing animal species would lead to functional expression. A BLAST search was performed on human EST data bases with the KCNK6 peptide sequence. Two sequences (EST accession numbers AA777982 and N39619) were identified corresponding to the P1-M2-M3-P2 region of the two-P domain channel subunits and were very close to KCNK6. The P2 domain showed a very unconventional sequence GLE instead of GLG found in TWIK-1 and KCNK6 pore domains. As for KCNK6, 5'- and 3'-RACE reactions allowed the identification of a start codon preceded by several stop codons. Interestingly, two 3'-RACE products were found containing the poly(A) tail. Sequencing revealed that both fragments are produced by splicing of a unique mRNA. Further analyses, detailed below, revealed that...
Exon skipping gives rise to five spliced mRNAs. These five mRNAs code for three protein forms, a subunit called KCNK7-A and two shorter variants called KCNK7-B and KCNK7-C with truncated cytoplasmic carboxyl termini. PCR was used to clone the complete coding sequences of KCNK7-A, KCNK7-B, and KCNK7-C from human brain cDNA. The independent cloning of these three forms confirmed sequences, and especially the peculiar sequence GLE found in the P2 domain. The KCNK7-A, KCNK7-B, and KCNK7-C cDNA have open reading frames of 921, 756, and 771 base pairs and code for proteins of 307, 252, and 257 amino acids (Fig. 5) with a calculated molecular mass of 32, 26, and 27 kDa, respectively. KCNK7-A has the hallmarks of a functional subunit of two-P domain potassium channels. KCNK7-A shows 80 and 42% amino acid identity, and 94 and 64% homology with the core region of KCNK6 and TWIK-1, respectively (Fig. 1). As expected, KCNK7-A is very close to KCNK6 (Fig. 5). Several sites are conserved between both subunits such as the consensus sites for N-linked glycosylation (residue 82) and the two sites for casein kinase II (residues 255 and 276). The short C-terminal tail of KCNK7-A also presents two potential Src homology 3 binding motifs. However, the EF-hand domain found in KCNK6 is not conserved. A site for Ca²⁺ calmodulin kinase (residue 8) at the cytoplasmic N-terminal tail could confer to KCNK7 an indirect regulation by Ca²⁺. The spliced forms, KCNK7-B and KCNK7-C, have shorter cytoplasmic carboxyl termini (Fig. 5). The M4 domain of KCNK7-B and KCNK7-C presents an abnormally short α-helical structure (about 16 residues as deduced from the nPHD program and hydropathy analyses (data not shown)) in comparison with the equivalent domains of the other K⁺ channels.

As previously indicated, the analysis of the variable 3' region by reverse transcription-PCR led us to the identification of five KCNK7 mRNA spliced forms (Fig. 6B). These different variants contain three alternative stop codons called stop-A, -B, and -C, as depicted in Fig. 6A. Sequencing of the different splice variants allowed us to identify the donor and acceptor splice sites (Fig. 6D). The longest protein form (KCNK7-A) is produced as the result of exclusion of an exon (exon skipping) containing an in-frame stop codon. The quantification of the different forms was performed by Southern blot and revealed that mRNAs coding for KCNK7-A, KCNK7-B, and KCNK7-C subunits are in proportions of 46, 39, and 8%, respectively (Fig. 6C). We cannot distinguish between KCNK7-B and KCNK7-C.
for the last 8% (Fig. 6, B and C). KCNK7 is expressed in the brain.

As for KCNK6, neither human KCNK7 nor its spliced forms are able to generate channel activity in transfected COS-7 cells. Attempts to record currents in cells transfected with all possible combinations of two of the KCNK6 and KCNK7-A, -B, and -C subunits also failed to produce any ion current.

Gene assignment by radiation hybrid panel mapping localized the human KCNK7 channel gene to chromosome 11q13 at 6.4 cRays from the framework marker WI-1409 (logarithm of odds score 21) (Fig. 7).

**DISCUSSION**

This paper reports the cloning of KCNK6 (mouse) and KCNK7 (human) subunits, which are structurally related to the growing family of K+ channels with four TMDs and two P domains (5–10). They are more particularly related to the TWIK-1 channel (40 and 42% amino acid identity, respectively). The previously cloned channels of this family all produce instantaneous and noninactivating currents, except TASK-2, which displays a relatively slow kinetic of activation. They are open at resting potential and are able to drive the membrane potential near the K+ equilibrium potential. There are three distinct functional subfamilies of two-P domain channels. TASK-1 and TASK-2 are background K+ channels sensitive to external pH variations near physiological pH (9, 10). Both TREK-1 (13) and TRAAK (6, 14) produce arachidonic acid-activated and mechanosensitive K+ currents, and TREK-1 but not TRAAK is inhibited by both protein kinase A and C (5). TWIK-1 is a weakly inward rectifying K+ channel that is stimulated by protein kinase C and inhibited by internal acidification (8). These K+ channels probably contribute in a major way to the regulation of the resting membrane potential and are probably endowed with a diversity of physiological roles.

Voltage-sensitive Ca2+-dependent K+ channels have been identified in many cell types and have now been cloned (40). Background Ca2+-activated K+ channels have also been re-
corded, but they have not yet been cloned in mammals. They are particularly well expressed in sensory neurons of the nodose ganglion, where they contribute to the lack of spontaneous activity observed in these cells (41). Background Ca\(^{2+}\)-activated K\(^{+}\)
channels have been cloned in plant cells and correspond to a two-P domain K\(^{+}\) channel, which is activated by cytosolic Ca\(^{2+}\) via two EF-hand domains (42). A reasonable assumption would then be that the mouse channel subunit KCNK6, which comprises an EF-hand domain, would also be a background channel regulated by internal Ca\(^{2+}\). Sequences of the human subunits KCNK7-A, -B, and -C do not contain an EF-hand domain, but an indirect Ca\(^{2+}\) regulation would also be possible in that case through the Ca\(^{2+}\)-calmodulin kinase site.

Electrophysiological experiments have revealed that neither KCNK6 nor KCNK7 can generate channel activity by themselves in Xenopus oocytes or transfected COS-7 cells. Reasons for this lack of expression have been particularly analyzed for KCNK6. Biochemical experiments showed KCNK6 protein expression and, as for TWIK-1 (11), subunit dimerization in transfected cells, indicating that the absence of channel activity was not due to a lack of expression or dimerization, or to a degradation of the protein. Immunocyto-localization on transfected COS-7 cells has shown that the absence of current is due to the fact that KCNK6 is unable to reach the plasma membrane. A first interpretation of this result would be that KCNK6 is an intracellular channel. If this were the case, then the putative K\(^{+}\) channel subunit would be expected to carry one of the signals for intracellular retention on its cytoplasmic regions. One such possible signal is indeed observed in a KCNK6 region that overlaps with the EF-hand region. However, suppression of this hypothetical retention signal by deletion did not confer access to the plasma membrane. Chimeras formed with KCNK6 and cytoplasmic elements of TREK1 or TASK1 channels (which have an easy access to the cell surface) also failed to reach the plasma membrane and to form electrophysiologically recordable channels. Another possibility, which is at present the most probable one, would be that KCNK6 needs a partner to reach the plasma membrane. However, this partner remains to be identified, and is not present in the heterologous expression systems used. An association between partner channel subunits essential for the expression of channel activity has been observed with G-protein-gated inward rectifiers (43, 44), with some of the voltage-sensitive K\(^{+}\) channels (17, 19, 45–47), or with epithelial Na\(^{+}\) channels (48–50).

**Fig. 6.** A, characterization of the exon skipping on the 3'-end of KCNK7 mRNA. B, agarose gel electrophoresis of PCR products obtained with primers F and R (see “Experimental Procedures”). This PCR was performed on human brain cDNA with (line 1) or without reverse transcriptase (line 3) and on human genomic DNA (line 2). The Southern blot (C) from line 1 is performed with probe 1 to realize a relative quantification of the different splicing forms by scanning (Tina program), and with probe 2 to show the skipped exon into the smaller band. The four bands in line 1 correspond to five splicing forms deduced from the sequencing of the different PCR products (D).

**Fig. 7.** Schematic diagram of human chromosome 11 and chromosomal location of human KCNK7 in the context of the Whitehead framework map. The Genebridge 4 radiation mapping panel (Research Genetics, Inc.) was used to map KCNK7 at 6.4 cRays from the framework marker WI-1409 with a logarithm of odds score for linkage of >21. Although radiation hybrid maps are not anchored to the cytogenetic maps, the most likely location of KCNK7 is 11q13.
generating the I-Ks cardiac K⁺ current (55–57).

KCNK6 is mainly expressed in tissues such as eyes, lung, and stomach. It is also significantly expressed in the embryo. The highest level of expression is found in the eyes, where in situ hybridization and immunohistochemistry showed that KCNK6 is only expressed in ganglion cells and in some neurons of the inner nuclear layer. This very restricted localization probably explains the absence of detection by Northern blot. In the mammalian retina, the first spontaneous Ca²⁺ waves are observed at postnatal day 2 and are thought to result from Ca²⁺ influx associated with burst of action potentials seen in ganglion cells at this developmental time (58–60). The early appearance of the KCNK6 channel in development, the fact that it has a Ca²⁺-binding domain probably conferring Ca²⁺ sensor properties, and its selective expression in ganglion cells suggest that this channel could play a role in the modulation of the electrical signal in the retina.

The human channel subunit KCNK7 is very close in structure from KCNK6 (80% of identity on the core channel), but it does not contain an EF-hand domain. KCNK7 RNA is alternatively spliced by an exon skipping mechanism, which generates five different mRNAs in the human brain. These five mRNAs present three alternative stop codons (named A, B, and C) and code for three subunits named KCNK7-A, -B, and -C. KCNK7-B and -C subunits are truncated in the C-terminal sequence. The mRNA coding for these two short forms represent about 50% of the total KCNK7 mRNA. Interestingly, KCNK7-B and -C have a very short M4 domain with a potential anchor at the C-terminal extremity (myristoylation site and amidation site, in KCNK7-B and KCNK7-C, respectively). Short TMDs are considered as important factors for the ER retention (27, 28, 61). Plasma membrane proteins generally have longer TMDs than Golgi membrane proteins (62). Therefore, the short M4 domain of KCNK7-B and -C could confer an intracellular localization.

The structures of the second P region (P2) of the different mammalian two-P domain channels are compared in Fig. 8. An important element of the signature of K⁺ channel function has long been recognized as being the P domain GYG sequence (63). This sequence is found in most voltage-sensitive, Ca²⁺-sensitive, and inward rectifier K⁺ channels (3, 64, 65). In the two-P domain channels, the canonical GYG structure is replaced by a GFG (TREK1, TASK1, TASK2, and TRAAK) or by a GLG (TWIK1 and KCNK6). A tryptophan residue (Trp213 in TWIK1) is conserved in TWIK1, TREK1, TASK1, TASK2, and TASK3, but it is replaced by a cysteine residue in KCNK6 and KCNK7. In addition, two adjacent residues, a tyrosine and a valine (Tyr234, Val235 in TWIK1), which are also present in all other members of the family, are replaced by a leucine-leucine sequence (Leu221-Leu222 in KCNK6) in KCNK6 and KCNK7. KCNK7 is unique, since a glutamic acid residue (Glu219 in KCNK7) is found instead of the strictly conserved Glu219 in KCNK6 and/or KCNK7 pore domain. Junctions between arrows indicate the singular amino acids found in the KCNK6 and/or KCNK7 pore domain. Junctions between arrows indicate the regions that are assumed to be close to each other in the tertiary structure (3). Bottom, pore model for the two-P domain channels, KCNK6 and KCNK7, based on the tertiary structure of the selectivity filter of the Kcsa⁺ channel (3).

FIG. 8. Top, alignment of the second pore region of mammalian two-P domain channels. The arrows indicate the singular amino acids found in the KCNK6 and/or KCNK7 pore domain. Bottom, pore model for the two-P domain channels, KCNK6 and KCNK7, based on the tertiary structure of the selectivity filter of the Kcsa⁺ channel (3).

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