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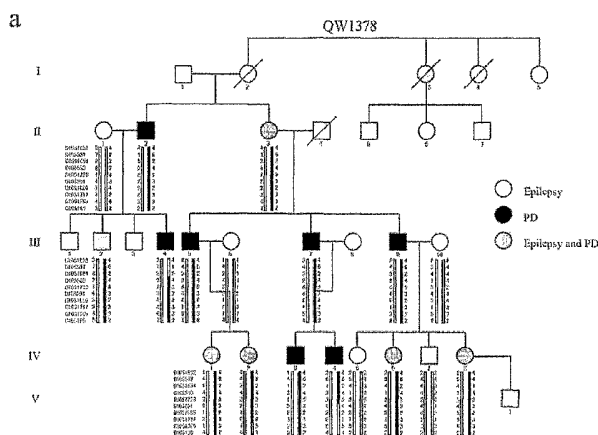
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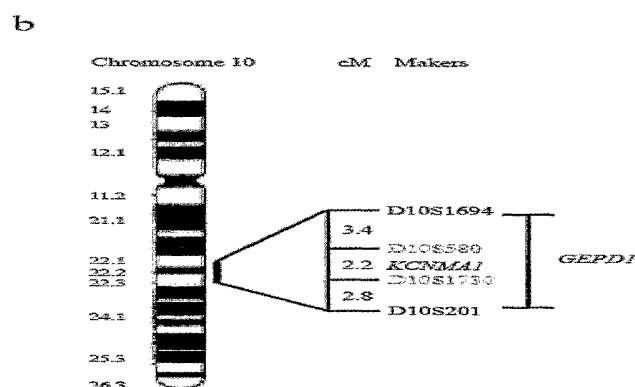
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(54) Title: PATHOGENIC GENE AND PROTEIN ASSOCIATED WITH PAROXYSMAL DYSKINESIA AND EPILEPSY



(57) Abstract: A method of identifying a patient that has, or is at risk of developing general epilepsy and/or paroxysmal dyskinesia includes detecting an alteration of the *KCNMA1* gene that disrupts the function of the BK channel so as to produce general epilepsy and/or paroxysmal dyskinesia.





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**PATHOGENIC GENE AND PROTEIN ASSOCIATED
WITH PAROXYSMAL DYSKINESIA AND EPILEPSY**

RELATED APPLICATION

Presently application claims priority from previous U.S. Provisional
5 Application Serial No. 60/628,676, filed November 17, 2004, which is herein
incorporated by reference in its entirety.

FIELD OF THE INVENTION

The present invention relates to pathogenic genes and proteins associated
with paroxysmal dyskinesia and epilepsy, and, more particularly, to genes and
10 proteins associated with co-existent paroxysmal dyskinesia and epilepsy.

BACKGROUND OF THE INVENTION

Epilepsy is one of the most common neurological disorders, affecting more
than 40 million people worldwide. It is defined by recurrent unprovoked seizures,
and increases risks of trauma and sudden death. Paroxysmal dyskinesias (PD) are a
15 heterogeneous group of neurological disorders characterized by recurrent brief
episodes of abnormal involuntary movements. PD can be broadly classified into
two main groups: paroxysmal kinesigenic dyskinesia (PKD) if the attacks are
induced by sudden movement and paroxysmal non-kinesigenic dyskinesia (PNKD)
if they were not. Although epilepsy and PD are two distinct disorders, the
20 co-existence of epilepsy and paroxysmal dyskinesia within the same individual is an
increasingly recognized neurological syndrome, with both sporadic and familial
cases described. Similarities in clinical features often make the two disorders
difficult to distinguish but also suggest shared pathophysiological mechanisms, such
as ion channel dysfunction. The basic underlying pathophysiology of PD, or the
25 co-occurrence of PD and epilepsy (PD/E), remains unknown. No specific genes
have been identified for PD or PD/E.

The large conductance voltage- and calcium-activated potassium channel
(BK, encoded by the *KCNMA1* gene) is a well-studied protein with wide expression

in many organs/tissues including central and peripheral neurons, but its *in vivo* physiological functions remain poorly defined.

SUMMARY OF THE INVENTION

The present invention relates to a pathogenic gene and protein encoded by the pathogenic gene for general epilepsy and/or paroxysmal dyskinesia (GEPD). It has been found that a mutation in the *KCNMA1* gene that results in a mutation of the human calcium-activated BK potassium channel alpha subunit (encoded by *KCNMA1*, or *Slo*) gene can cause GEPD. Mutations of the *KCNMA1* gene can include nucleotide additions, substitutions, or deletions relative to the nucleotide sequence of this gene that result in an increase in the calcium sensitivity and enhancement of BK channel encoded by the *KCNMA1* gene (i.e., the BK channel or MaxiK channel). Enhancement of BK channels *in vivo* can lead to increased excitability by inducing rapid repolarization of action potentials, resulting in GEPD and by allowing neurons to fire at a faster rate.

Accordingly, one aspect of the present invention is directed to a method of identifying a patient that has, or is at risk of developing general epilepsy and/or paroxysmal dyskinesia by determining whether the *KCNMA1* gene is mutated. Mutations of the *KCNMA1* gene can include nucleotide additions, substitutions, or deletions relative to the nucleotide sequence of this gene that result in an increase in the calcium sensitivity and enhancement of BK channel encoded by the *KCNMA1* gene. Patients identified by this method can be those that are exhibiting clinical characteristics suggesting that they may have general epilepsy and/or paroxysmal dyskinesia or individuals that do not exhibit clinical characteristics or symptoms of paroxysmal dyskinesia and/or epilepsy. In the first case, the method can be used to make or confirm diagnosis of paroxysmal dyskinesia and/or epilepsy and, in the latter case, the method can be used to predict whether the patient or their offspring are likely to develop or are vulnerable to the paroxysmal dyskinesia and/or epilepsy.

In general, the likelihood of a patient having or developing paroxysmal dyskinesia and/or epilepsy is dependent on the particular genetic mutation. Genetic mutations that result in an increase in the calcium sensitivity and enhancement of BK channel can trigger the pathogenesis of general epilepsy and/or paroxysmal dyskinesia in a patient. For example, patients having a heterozygous A→G transition in exon 10 of the *KCNMA1* gene that results in the substitution of a negatively charged aspartic acid residue for a neutral glycine residue (D434G) in the RCK domain (regulator of conductance for K⁺) were found to have GEPD. The mutant D434G BK channel displayed a larger macroscopic current at a given voltage and calcium concentration and a faster activation and rate with depolarizing voltage steps.

The extent to which the *KCNMA1* gene has been mutated can be determined by any means including direct nucleotide analysis or hybridization under conditions selected to reveal mutations. One preferred method is to amplify one or more regions of the relevant gene using polymerase chain reaction (PCR) and to then analyze the amplification products, for example, by sequence analysis, heteroduplex analysis, or single strand conformational polymorphism analysis. In this aspect of the invention, the region amplified corresponds to exon 10 of the *KCNMA1* gene.

A further aspect of the invention relates to a method of identifying a patient that has, or is likely to develop paroxysmal dyskinesia and/or epilepsy by determining if a BK channel is mutated. The mutation to the BK channel can include amino acid additions, substitutions, or deletions that result in an increase in calcium sensitivity of the BK channel and that can trigger the pathogenesis of paroxysmal dyskinesia and/or epilepsy in a patient. Mutations in the BK channel can be detected by methods, such as enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations, and immunofluorescence.

Another aspect of the invention relates to an isolated polynucleotide encoding a mutant or variant α -subunit of a human BK channel, which can cause

GEPD. The isolated nucleic acid can cause a mutation of the RCK domain of the α -subunit of a human BK channel, which can result in the α -subunit of a human BK channel having an abnormal (e.g., elevated) affinity for calcium. In one example, the isolated nucleic acid can have a nucleic acid sequence corresponding to SEQ ID NO: 2.

Yet another aspect of the invention relates to an isolated polypeptide that is a mutant or variant α -subunit of a human BK channel, which can cause GEPD. The isolated polypeptide includes a mutation of the RCK domain, which can result in polypeptide having an abnormal (e.g., elevated) affinity for calcium. In one example, the isolated polypeptide can have an amino acid sequence corresponding to SEQ ID NO: 4.

The present invention also relates to methods of using the isolated polynucleotides and polypeptides to identify compounds that are useful in the treatment and diagnosis of GEPD. The compounds can act as agonists or antagonists of mutant or variant expression or function. The polynucleotides and polypeptides serve as both a target to identify compounds and may themselves provide a source for derivative compounds that can act as an agonist or antagonist of mutant *KCNMA1* expression or function. The invention is further directed to using these compounds to treat and diagnose GEPD. In one embodiment, methods are directed to treating cells, tissues, or animal models associated with the disorder using the *KCNMA1* gene or gene product as a reagent or target for treatment.

The invention is thus also directed to methods of using the mutant *KCNMA1* gene or polypeptide as a reagent or target to screen for agents that modulate the levels or effectively reverse the mutation or other abnormality in the *KCNMA1* gene. For example, the agents can comprise antibody, which is immunologically, reactive with an isolated polypeptide. Accordingly, the invention provides methods for identifying agonists and antagonists of the *KCNMA1* gene. These agents can be used to treat GEPD by their effects on the level or function of the *KCNMA1* gene or

gene product. By identifying agents that are capable of modulating the expression or function of the *KCNMA1* gene or gene product, methods are thus provided for affecting the development of or course of GEPD in an individual by modulating the level. Further, by providing these agents that modulate the expression, methods are provided for assessing the effect of treatment in cell and animal models.

By identifying agents that are capable of interacting with, or otherwise allowing detection of abnormal expression or function of the *KCNMA1* gene or gene product, methods are thus provided for diagnosing the development of, or risk of developing, GEPD. This can be in the context of an individual patient, monitoring clinical trials, and assessing *KCNMA1* gene function or efficacy of treatment in cell and animal models. The invention also provides cell and animal model systems for studying GEPD based on alterations in the *KCNMA1* gene or gene product in the model.

The invention is further directed to methods of using the mutant *KCNMA1* gene or polypeptide as a reagent or target to screen for agents that can treat GEPD based on alterations in the *KCNMA1* gene or gene product. The alterations can result in an increase in the calcium sensitivity and enhancement of BK channel encoded by the *KCNMA1* gene (i.e., the BK channel or MaxiK channel). These agents can comprise compounds that block or modulate voltage sensitive Ca^{2+} -activated K^{+} channels (e.g., BK channel or MaxiK channel). Examples of such compounds can include potassium channel blockers, such as paxilline, charybdotoxin, iberiotoxin, penitrem A, tetraethyl ammonium chloride (TEA), apamin, clonitrimazole, dequalium chloride, iberiotoxin, neuropeptide Y, tityustoxin. In one embodiment, methods are directed to treating cells, tissues, or animal models comprising the mutant *KCNMA1* gene or gene product.

BRIEF DESCRIPTION OF THE DRAWINGS

Further features of the present invention will become apparent to those skilled in the art to which the present invention relates from reading the following description of the invention with reference to the accompanying drawings in which:

5 Fig. 1 illustrates the genetic linkage of GEPD to chromosome 10q22 (*GEPD1*). (a) Pedigree structure and genotypic analysis of the family affected with epilepsy, PD, or both. Affected individuals are indicated by filled squares (males) or circles (females), and unaffected individuals shown as empty symbols. The deceased individuals are indicated using slashes (/). Genotypes for markers
10 D10S1652, D10S537, D10S1694, D10S580, D10S1730, D10S201, D10S1686, D10S1717, D10S1765, and D10S185 and results of haplotype analysis are shown below each individual. The haplotype that co-segregates with the disease is indicated by a black vertical bar. (b) Ideogram of chromosome 10 with the Geimsa banding patterns and localization of the first GEPD locus (*GEPD1*). The genetic
15 map with the location of the *GEPD1* gene (*KCNMA1*) is shown.

 Fig. 2 illustrates the *KCNMA1* mutation D434G cosegregates with GEPD patients in kindred QW1378. (a) DNA sequence analysis of exon 10 of *KCNMA1* from a normal individual (III-10) and the proband (IV-8) revealed an A→G substitution (reverse sequence) at codon 434 in the proband. The A→G substitution
20 results in the replacement of a negatively charged aspartic acid residue D434 by a neutral amino acid glycine, D434G. (b) Structure of the BK channel with the GEPD mutation D434G indicated. (c) The D434 residue of *KCNMA1* is evolutionally conserved among different species. (d) *KCNMA1* mutation D434G co-segregates with GEPD patients in the family. Pedigree showing clinical status is shown on the
25 top. The results of RFLP analysis of mutation D434G using a 2% agarose gel are shown below each individual. Wild type allele, 201 bp; mutant allele, 83 bp + 118 bp.

Fig. 3 illustrates electrophysiological characterization of wild type and mutant D434G *KCNMA1* potassium channels in *Xenopus* oocytes. (a) Selected current traces of WT (left) and mutant D434G (right) channels at 2 μM $[\text{Ca}^{2+}]_i$. Test potential were -10 to +140 mV with 50-mV increments. The holding and repolarizing potentials were -80 and -50 mV. (b) Mean G-V relations of WT and mutant D434G channels at 0.1 and 2 μM $[\text{Ca}^{2+}]_i$. All the G-V relations are fitted with the Boltzmann relation (solid lines) with $V_{1/2}$ and slope factor at 2 μM $[\text{Ca}^{2+}]_i$: 116 ± 5 mV, 20.6 ± 4.4 for WT and 58.9 ± 4.8 mV, 17.6 ± 4.3 for D434G; and at 0.1 μM $[\text{Ca}^{2+}]_i$: 184 ± 8 mV, 20.5 ± 6.3 for WT and 157 ± 5 mV, 20.3 ± 4.2 for D434G. (c) Plots of activation time constants of WT and mutant D434G channels as a function of test potential at 0.1 and 2 μM $[\text{Ca}^{2+}]_i$. The curves are fitted with an exponential function (solid lines).

Fig. 4 illustrates the D434G mutation of *KCNMA1* potassium channels caused an increase in open channel probability (Popen), consistent with an increase in sensitivity to calcium. (a) Single-channel currents recorded from WT (left) and D434G *KCNMA1* channels (right) expressed in CHO cells. In 5 μM (upper traces) and 10 μM (lower traces) intracellular free Ca^{2+} , the mutated channel was more likely to be in the open state. There was no difference in single channel conductance. c - closed state; o - open state; dotted lines - zero current level. Membrane potential +80 mV. (b) Effect of membrane potential on Popen of WT and mutant D434G channels at three calcium concentrations (2, 10 and 50 μM). Lines are fits to the Boltzmann equation. (c) Relationship between intracellular free $[\text{Ca}^{2+}]_i$, membrane potential, and Popen. Each line is the Boltzmann fit obtained as in part b. The difference between curves becomes less at high $[\text{Ca}^{2+}]_i$, consistent with saturation of calcium binding. (d) Relationship between Popen and $[\text{Ca}^{2+}]_i$ at membrane potentials of +60 mV (circles) and +80 mV (triangles) in WT and mutant D434G channels. Lines are fits to the Hill equation. For b-d, solid lines are WT and dashed lines are the mutant D434G channels. For b and d, solid symbols are WT, open

symbols are the mutant D434G channels, $n = 5$ to 17 patches for each point and error bars are SEM.

Fig. 5 illustrates a representative interictal EEG of an affected family member of kindred QW1378. Ten-second EEG tracing demonstrating interictal
5 generalized spike-wave complexes in an individual affected with both generalized epilepsy and paroxysmal dyskinesia.

DETAILED DESCRIPTION

The present invention relates to a novel genetic locus for a pathogenic gene and a protein encoded by the pathogenic gene for general epilepsy and/or
10 paroxysmal dyskinesia (GEPD). It has been found that a mutation or variation in the *KCNMA1* gene located on the 10q22 chromosome, which results in a mutation of the human calcium-activated BK potassium channel alpha subunit (encoded by *KCNMA1*, or *Slo*) gene, can cause GEPD. By way of example, a heterozygous A→G transition was identified in exon 10 of the *KCNMA1* gene in the proband
15 (IV-8) of a family. Exon 10 of the *KCNMA1* gene has a wild type nucleotide sequence that corresponds with SEQ ID NO: 1. The mutation of exon 10 of the *KCNMA1* gene has nucleotide sequence that corresponds with SEQ ID NO: 2. The A→G transition results in the substitution of a negatively charged aspartic acid residue for a neutral glycine residue (D434G) in the RCK domain (regulator of
20 conductance for K^+). The calcium-activated BK potassium channel alpha subunit has an amino acid sequence that corresponds with SEQ ID NO: 3, and the mutated calcium-activated BK potassium channel alpha subunit has an amino acid sequence that corresponds with SEQ ID NO: 4.

The BK channel is activated by both membrane depolarization and a rise in
25 cytosolic Ca^{2+} . The pore-forming α -subunit contains seven transmembrane domains (S0-S6) at the N terminus and an extensive C terminus with four hydrophobic segments (S7-S10) and the Ca^{2+} bowl. Between S6 and S8 is the RCK domain, which may contain binding sites for a variety of regulatory ligands,

including Ca^{2+} and Mg^{2+} . As the mutation D434G is located in the RCK domain, it is believed that the mutation may cause abnormal calcium affinity of the BK channel. Mutations of the α -subunit that increase Ca^{2+} sensitivity of BK channels have not been previously reported. An increase in calcium sensitivity of the BK channel
5 would lead to greater macroscopic potassium conductance under physiological conditions. Thus, the D434G mutation leads to gain-of-function of the α -subunit.

Gain-of-function of the BK channel can lead to an increase in brain excitability, causing generalized epilepsy when the thalamus or thalamo-cortical circuits are involved, and paroxysmal dyskinesia when the basal ganglia is involved.
10 The most likely mechanism relates to a more rapid repolarization of action potentials by the mutant D434G channels. Enhancing this repolarization can enable faster repriming (removal of inactivation) of sodium channels, and thus allow neurons to fire at a higher frequency. Additionally, enhancing some inhibitory currents can switch neurons within a circuit into a bursting mode, as can occur with absence
15 seizures that depend upon activation of inhibitory GABA_B receptors within the thalamus. Likewise, gain-of-function of BK channels can lead to greater hyperpolarization and activate the hyperpolarization-activated cation current, I_h , resulting in generation of secondary depolarizations.

One aspect of the present invention is therefore directed to methods of using
20 the *KCNMA1* or gene products as a target to detect GEPD or the risk of developing GEPD. The invention is also directed to methods for determining the molecular basis GEPD or the risk of GEPD using the *KCNMA1* gene or gene products as a target. It is understood that "gene product" refers to all molecules derived from the gene, especially RNA and protein. cDNA is also encompassed, where, for example,
25 made by naturally-occurring reverse transcriptase.

In an aspect of the invention, the method includes detecting the *KCNMA1* gene itself and/or alterations in copy number, genomic position, and nucleotide sequence of the *KCNMA1* gene. Alterations in the *KCNMA1* nucleotide sequence

include the insertion, deletion, point mutation, and inversion of nucleic acids of nucleotide sequence. The alterations can occur at any position within the gene, including coding, noncoding, transcribed, and non-transcribed, regulatory regions. For example, the alteration can be a heterozygous A→G transition in exon 10 of the *KCNMA1* gene that results in a gain of function of the BK channel. Other alterations that can be detected include nucleic acid modification, such as methylation, gross rearrangement in the genome, such as in a homogeneously-staining region, double minute chromosome or other extrachromosomal element, or cytoskeletal arrangement.

The present invention also encompasses the detection of RNA transcribed from the *KCNMA1* gene. Detection of the RNA transcribed from the *KCNMA1* gene encompasses alterations in copy number and nucleotide sequence. Sequence changes include insertions, deletions, point mutations, inversions, and splicing variations. Detection of *KCNMA1* RNA can be indirectly accomplished by means of its cDNA.

KCNMA1 DNA and RNA levels and gross rearrangement can be analyzed by any of the standard methods known in the art. In such methods, nucleic acid can be isolated from a cell or analyzed *in situ* in a cell or tissue sample. For detecting alterations in nucleic acid levels or gross rearrangement, all, or any part, of the nucleic acid molecule can be detected. Nucleic acid reagents derived from any desired region of the *KCNMA1* gene can be used as a probe or primer for these procedures. Copy number can be assessed by *in situ* hybridization or isolation of nucleic acid from the cell and quantitation by standard hybridization procedures such as Southern or Northern analysis. Genes can be amplified in the forms of homogeneously-staining regions or double minute chromosomes. Accordingly, one method of detection involves assessing the cellular position of an amplified gene. This method encompasses standard *in situ* hybridization methods, or alternatively,

detection of an amplified fragment derived from digestion with an appropriate restriction enzyme recognizing a sequence that is repeated in the amplified unit.

Identifying nucleic acid modifications, such as methylation, can be analyzed by any of the known methods in the art for digesting nucleic acid and analyzing modified nucleotides, such as by BPLC, thin-layer chromatography, mass spectra analysis, and the like. Gross rearrangements in the genome are preferably detected by means of *in situ* hybridization, although this type of alteration can also be assessed by means of assays involving normal cellular components with which the genes are normally found, such as in specific membrane preparations.

Mutations in *KCNMA1* nucleic acids can be analyzed by any of the standard methods known in the art. Nucleic acid can be isolated from a cell or analyzed *in situ* in a cell or tissue sample by means of specific hybridization probes designed to allow detection of the mutation. The portion of the nucleic acid that is detected preferably contains the mutation. It is to be understood that in some embodiments, as where the mutation affects secondary structure or other cellular association, distant regions affected by the mutation can be detected. The nucleic acid reagents can be derived from the mutated region of the *KCNMA1* gene to be used as a probe or primer for the procedures. However, as discussed above, nucleic acid reagents useful as probes can be derived from any position in the nucleic acid. RNA or cDNA can be used in the same way.

In certain aspects of the invention, detection of the mutation involves the use of a probe/primer in a polymerase chain reaction (PCR) (see, e.g. U.S. Pat. Nos. 4,683,195 and 4,683,202), such as anchor PCR or RACE PCR, or, alternatively, in a ligation chain reaction (LCR) (see, e.g., Landegran et al., Science 241:1077-1080 (1988); and Nakazawa et al., PNAS 91:360-364 (1994)), the latter of which can be particularly useful for detecting point mutations in the gene (see Abravaya et al., Nucleic Acids Res. 23:675-682 (1995)). This method can include the steps of collecting a sample of cells from a patient, isolating nucleic acid (e.g., genomic,

mRNA or both) from the cells of the sample, contacting the nucleic acid sample with one or more primers which specifically hybridize to a gene under conditions such that hybridization and amplification of the gene (if present) occurs, and detecting the presence or absence of an amplification product, or detecting the size of the amplification product and comparing the length to a control sample (*e.g.*, a wild-type *KCNMA1* nucleic acid). Deletions and insertions can be detected by a change in size of the amplified product compared to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to normal (or wild-type) RNA or antisense DNA sequences.

Alternatively, mutations in a *KCNMA1* gene can be directly identified, for example, by alterations in restriction enzyme digestion patterns determined by gel electrophoresis. Further, sequence-specific ribozymes can be used to score for the presence of specific mutations by development or loss of a ribozyme cleavage site. Perfectly matched sequences can be distinguished from mismatched sequences by nuclease cleavage digestion assays or by differences in melting temperature. Sequence changes at specific locations can also be assessed by nuclease protection assays such as RNase and SI protection or the chemical cleavage method. Furthermore, sequence differences between a mutant *KCNMA1* gene and a wild-type gene can be determined by direct DNA sequencing. A variety of automated sequencing procedures can be utilized when performing the diagnostic assays ((1995) *Biotechniques* 19:448), including sequencing by mass spectrometry (*e.g.*, PCT International Publication No. WO 94/16101; Cohen et al., *Adv. Chromatogr.* 36:127-162 (1996); and Griffin et al., *Appl. Biochem. Biotechnol.* 38:147-159 (1993)).

Other methods for detecting mutations in the gene include methods in which protection from cleavage agents is used to detect mismatched bases in RNA/RNA or RNA/DNA duplexes (Myers et al., *Science* 230:1242 (1985)); Cotton et al., *PNAS* 85:4397 (1988); Saleeba et al., *Meth. Enzymol.* 217:286-295 (1992)),

electrophoretic mobility of mutant and wild type nucleic acid is compared (Orita et al., PNAS 86:2766 (1989); Cotton et al., Mutat. Res. 285:125-144 (1993); and Hayashi et al., Genet. Anal. Tech. Appl. 9:73-79 (1992)), and movement of mutant or wild-type fragments in polyacrylamide gels containing a gradient of denaturant is
5 assayed using denaturing gradient gel electrophoresis (Myers et al., Nature 313:495 (1985)). Examples of other techniques for detecting point mutations include, selective oligonucleotide hybridization, selective amplification, and selective primer extension.

Methods of detection of a mutation of the *KCNMA1* gene can also include
10 detection of the *KCNMA1* protein (the BK channel α -subunit) encoded by the *KCNMA1* gene. Detection encompasses assessing protein levels, mutation, post-translational modification, and subcellular localization. Mutations encompass deletion, insertion, substitution and inversion. Mutations at RNA splice junctions can result in protein splice variants.

15 The BK channel α -subunit protein levels can be analyzed by any of the standard methods known in the art. BK channel α -subunit protein can be isolated from the cell or analyzed *in situ* in a cell or tissue sample. Quantification of the BK channel α -subunit protein can be accomplished *in situ*, for example by standard of fluorescence detection procedures involving a fluorescently labeled binding partner,
20 such as an antibody or other protein with which the BK channel α -subunit protein will bind. This could include a substrate upon which the protein acts or an enzyme, which normally acts on the protein. Quantification of isolated protein can be accomplished by other standard methods for isolated protein, such as *in situ* gel detection, Western blot, or quantitative protein blot. Levels can also be assayed by
25 functional means, such as the effects upon a specific substrate. In the case of the BK channel α -subunit protein, this could involve the cleavage of basic amino acids from the C-terminus of the various peptide substrates upon, which the BK channel α -subunit protein normally acts, or artificial substrates designed for this assay. It is

understood that any enzyme activity contained in the BK channel α -subunit protein can be used to assess protein levels.

Mutations in the BK channel α -subunit can be analyzed by any of the above or other standard methods known in the art. Protein can be isolated from the cell or analyzed *in situ* in a cell or tissue sample. Analytic methods include assays for altered electrophoretic mobility, binding properties, tryptic peptide digest, molecular weight, antibody-binding pattern, isoelectric point, amino acid sequence, and any other of the known assay techniques useful for detecting mutations in a protein. Assays include, but are not limited to, those discussed in Varlamov et al., J. Biol. Chem. 271:13981 (1996), incorporated herein by reference for teaching such assays. These include C-terminal arginine binding, acidic pH optima, sensitivity to inhibitors, thermal stability, intracellular distribution, endopeptidase activity, effect on endopeptidase inhibitor, substrate affinity, enzyme kinetics, membrane association, posttranslational modification, active site confirmation, compartmentalization, binding to substrate, secretion, and turnover. Further assays for function can be found in Fricker, J. Cell Biochem. 38:279-289 (1988), and Manser et al., Biochem. J. 267:517-525, (1990), both incorporated by reference for teaching specific functions that can be assayed for mutation in the *KCNMA1* gene.

In vitro techniques for detection of the protein include enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations and immunofluorescence. Alternatively, the protein can be detected *in vivo* in a subject by introducing into the subject a labeled anti- BK channel α -subunit antibody. For example, the antibody can be labeled with a radioactive marker whose presence and location in a subject can be detected by standard imaging techniques. For detection of specific mutation in the protein, antibodies, or other binding partners, can be used that specifically recognize these alterations. Alternatively, mutations can be detected by direct sequencing of the protein.

Other alterations that can be detected include alterations in post-translational modification. Amino acids, including the terminal amino acids, may be modified by natural processes, such as processing and other post-translational modifications. Common modifications that occur naturally in polypeptides are described in basic texts, detailed monographs, and the research literature, and they are well known to those of skill in the art.

Known modifications include, but are not limited to, acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent crosslinks, formation of cystine, formation of pyroglutamate, formylation, gamma carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination.

Such modifications are well-known to those of skill in the art and have been described in great detail in the scientific literature. Several particularly common modifications, glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation, for instance, are described in most basic texts, such as *Proteins--Structure and Molecular Properties*, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York (1993). Many detailed reviews are available on this subject, such as by Wold, F., *Posttranslational Covalent Modification of Proteins*, B. C. Johnson, Ed., Academic Press, New York 1-12 (1983); Seifter et al. (*Meth. Enzymol.* 182: 626-646 (1990)) and Rattan et al. (*Ann. N.Y. Acad. Sci.* 663:48-62 (1992)).

In addition to detection methods that involve specific physical features, functional characteristics of the BK channel α -subunit are also useful for detection with known methods. These include changes in biochemistry, such as substrate affinity, enzyme kinetics, membrane association, active site conformation, compartmentalization, forming a complex with substrates or enzymes that act upon the protein, secretion, turnover, pH optima, sensitivity to inhibitors, thermal stability, endopeptidase activity, effects on endopeptidase inhibitors, and any other such functional characteristic that is indicative of a mutation or alteration in post-translational modification. Specific assays can be found in the literature (*e.g.*, see Varlamov et al. (1996) J. Biol. Chem. 271:13981).

KCNMA1 gene and gene product can be detected in a variety of systems. These include cell-free and cell-based systems *in vitro*, tissues, such as *ex vivo* tissues for returning to a patient, in a biopsy, and *in vivo*, such as in patients being treated, for monitoring clinical trials, and in animal models. Cell-free systems can be derived from cell lines or cell strains *in vitro*, including recombinant cells, cells derived from patients, subjects involved in clinical trials, and animal models, including transgenic animal models. In one embodiment, *KCNMA1* gene and gene product can also be detected in cell-based systems. This includes cell lines and cell strains *in vitro*, including recombinant lines and strains containing the *KCNMA1* gene, expanded cells, such as primary cultures, particularly those derived from a patient with GEPD, subjects undergoing clinical trials, and animal models of GEPD including transgenic animals. The *KCNMA1* gene and gene product can also be detected in tissues. These include tissues derived from patients with GEPD, subjects undergoing clinical trials, and animal models. In one embodiment, the tissues are those affected in GEPD (*e.g.*, neural tissue). The *KCNMA1* gene and gene product can also be detected in individual patients with GEPD, and subjects undergoing clinical trials, and in animal models of GEPD, including transgenic models.

Preferred sources of detection include cell and tissue biopsies from individuals affected with GEPD or at risk for developing GEPD.

In addition to detecting the *KCNMA1* gene or gene products directly, the invention also encompasses the use of compounds that produce a specific effect on a variant *KCNMA1* gene or gene product as a further means of diagnosis. This includes, for example, detection of binding partners, including binding partners specific for variant *KCNMA1* genes or gene products, and compounds that have a detectable effect on a function of *KCNMA1* genes or gene products.

All these methods of detection can be used in procedures to screen individuals at risk for developing or having GEPD. Further, detection of the alterations of the gene or gene products in individuals can serve as a prognostic marker for developing GEPD or a diagnostic marker for having GEPD when the individuals are not known to have GEPD or to be at risk for having GEPD. Diagnostic assays can be performed in cell-based systems, and particularly in cells associated with GEPD, in intact tissue, such as a biopsy, and nonhuman animals and humans *in vivo*. Diagnosis can be at the level of nucleic acid or polypeptide.

The invention also encompasses methods for modulating the level or activity of *KCNMA1* gene or gene products. At the level of the gene, known recombinant techniques can be used to alter the gene *in vitro* or *in situ*. Excessive copies of, or all or part of, the *KCNMA1* gene can be deleted. Deletions can be made in any desired region of the gene including transcribed, non-transcribed, coding and non-coding regions. Additional copies of part or all of the gene can also be introduced into a genome. Finally, alterations in nucleotide sequence can be introduced into the gene by recombinant techniques. Alterations include deletions, insertions, inversions, and point mutation. Accordingly, GEPD that is caused by a mutated *KCNMA1* gene could be treated by introducing a functional (wild-type) *KCNMA1* gene into the individual. Further, specific alterations could be introduced into the gene and function tested in any given cell type, such as in cell-based models for GEPD. Still

further, any given mutation can be introduced into a cell and used to form a transgenic animal, which can then serve as a model for GEPD testing.

Homologously recombinant host cells can also be produced that allow the *in situ* alteration of endogenous *KCNMA1* polynucleotide sequences in a host cell genome. This technology is more fully described in U.S. Pat. No. 5,641,670, which is herein incorporated by reference. Briefly, specific polynucleotide sequences corresponding to the *KCNMA1* polynucleotides or sequences proximal or distal to a *KCNMA1* gene are allowed to integrate into a host cell genome by homologous recombination where expression of the gene can be affected. In one embodiment, regulatory sequences are introduced that either increase or decrease expression of an endogenous sequence.

The levels and activity of *KCNMA1* RNA are also subject to modulation. Polynucleotides corresponding to any desired region of the RNA can be used directly to block transcription or translation of *KCNMA1* sequences by means of antisense or ribozyme constructs. Thus, where the disorder is characterized by abnormally high gene expression, these nucleic acids can be used to decrease expression levels. A DNA antisense polynucleotide is designed to be complementary to a region of the gene involved in transcription, preventing transcription and hence production of protein. An antisense RNA or DNA polynucleotide would hybridize to the mRNA and thus block translation of mRNA into protein. An alternative technique involves cleavage by ribozymes containing nucleotide sequences complementary to one or more regions in the mRNA that attenuate the ability of the mRNA to be translated.

The present invention also includes the modulation of nucleic acid expression using compounds that have been discovered by screening the effects of the compounds on *KCNMA1* nucleic acid levels or function.

The invention is further directed to methods for modulating BK channel protein levels or function. For example, antibodies can be prepared against specific

fragments containing sites required for function or against the intact protein. Protein levels can also be modulated by use of compounds discovered in screening techniques in which the protein levels serve as a target for effective compounds. Finally, mutant BK channel proteins can be functionally affected by the use of compounds discovered in screening techniques that use an alteration of mutant function as an end point.

Modulation can be in a cell-free system. In this context, for example, the assay could involve cleavage of substrate or other indicator of *KCNMA1* activity. Modulation can also occur in cell-based systems. These cells may be permanent cell lines, cell strains, primary cultures, recombinant cells, cells derived from affected individuals, and transgenic animal models of CAD or MI, among others. Modulation can also be *in vivo*, for example, in patients having the disorder, in subjects undergoing clinical trials, and animal models of GEPD, including transgenic animal models. Modulation could be measured by direct assay of the *KCNMA1* gene or gene product or by the results of *KCNMA1* gene and gene product function. All of these methods can be used to affect *KCNMA1* function in individuals having or at risk for having GEPD. Thus, the invention encompasses the treatment of GEPD by modulating the levels or function of *KCNMA1* genes or gene product.

The invention also encompasses methods for identifying compounds that interact with the *KCNMA1* gene or gene product, particularly to modulate the level or function of the *KCNMA1* gene or gene product. Modulation can be at the level of transcription, translation, or polypeptide function. Accordingly, where levels of *KCNMA1* gene or gene product are abnormally high or low, compounds can be screened for the ability to correct the level of expression. Alternatively, where a mutation affects the function of the *KCNMA1* nucleic acid or protein, compounds can be screened for their ability to compensate for or to correct the dysfunction. In this manner, *KCNMA1* and *KCNMA1* variants can be used to identify agonists and

antagonists useful for affecting *KCNMA1* and variant gene expression. These compounds can then be used to affect *KCNMA1* expression or function in individuals with GEPD. Thus, these screening methods are useful to identify compounds that can be used for treating GEPD.

5 These compounds are also useful in a diagnostic context in that they can then be used to identify altered levels of *KCNMA1* or *KCNMA1* variants in a cell, tissue, nonhuman animal, and human. For example, compounds specifically interacting with *KCNMA1* nucleic acid or protein to produce a particular result, by producing that result in a cell, tissue, nonhuman animal, or human, indicate that there is a lesion
10 in the *KCNMA1* gene or gene product.

 Thus, modulators of gene expression can be identified in a method wherein *KCNMA1* gene or gene product is contacted with a candidate compound and the level or expression of gene or gene product is determined. The level or expression of gene or gene product in the presence of the candidate compound is compared to the
15 level or expression of gene or gene product in the absence of the candidate compound. The candidate compound can then be identified as a modulator of nucleic acid or protein expression based on this comparison and be used, for example, to treat GEPD. When the level or expression of gene or gene product is statistically significantly greater in the presence of the candidate compound than in
20 its absence, the candidate compound is identified as a stimulator of levels or expression of the gene or gene product. When levels or product expression are statistically significantly less in the presence of the candidate compound than in its absence, the candidate compound is identified as an inhibitor.

 These compounds can be used to test on model systems, including animal
25 models of GEPD, and human clinical trial subjects, cells derived from these sources as well as transgenic animal models of GEPD. Accordingly, the present invention provides methods of treatment, with the gene or gene product as a target, using a compound identified through drug screening as a modulator to modulate expression

of the gene or gene product. Modulation includes both up-regulation (*i.e.*, activation or agonization) or down-regulation (*i.e.*, suppression or antagonization) or nucleic acid expression.

Further, the expression of genes that are up- or down-regulated in response to *KCNMA1* can also be assayed. In this embodiment the regulatory regions of these genes can be operably linked to a reporter gene. Candidate compounds include, for example, 1) peptides, such as soluble peptides, including Ig-tailed fusion peptides and members of random peptide libraries (see, *e.g.*, Lam et al., Nature 354:82-84 (1991); Houghten et al., Nature 354:84-86 (1991)) and combinatorial chemistry-derived molecular libraries made of D- and/or L-configuration amino acids; 2) phosphopeptides (*e.g.*, members of random and partially degenerate, directed phosphopeptide libraries, see, *e.g.*, Songyang et al., Cell 72:767-778 (1993)); 3) antibodies (*e.g.*, polyclonal, monoclonal, humanized, anti-idiotypic, chimeric, and single chain antibodies as well as Fab, F(ab')₂, Fab expression library fragments, and epitope-binding fragments of antibodies); and 4) small organic and inorganic molecules (*e.g.*, molecules obtained from combinatorial and natural product libraries).

Any of the biological or biochemical functions mediated by *KCNMA1* can be used in an endpoint assay. These include all of the biochemical or biochemical/biological events described herein, in the references cited herein, incorporated by reference for these endpoint assay targets, and other functions known to those of ordinary skill in the art.

The invention is further directed to methods of using the mutant *KCNMA1* gene or polypeptide as a reagent or target to screen for agents that can treat GEPD based on alterations in the *KCNMA1* gene or gene product. The alterations can result in an increase in the calcium sensitivity and enhancement of BK channel encoded by the *KCNMA1* gene (*i.e.*, the BK channel or MaxiK channel). These agents can comprise compounds that block or modulate voltage sensitive

Ca²⁺-activated K⁺ channels (e.g., BK channel or MaxiK channel). Examples of such compounds can include potassium channel blockers, such as paxilline, charybdotoxin, iberiotoxin, penitrem A, tetraethyl ammonium chloride (TEA), apamin, clortrimazole, dequalium chloride, iberiotoxin, neuropeptide Y, tityustoxin.

5 In one embodiment, methods are directed to treating cells, tissues, or animal models associated with the disorder using the *KCNMA1* gene or gene product as a reagent or target for treatment.

A further aspect of the invention involves pharmacogenomic analysis in the case of polymorphic *KCNMA1* proteins and specific mutants. Pharmacogenomics
10 deal with clinically significant hereditary variations in the response to drugs due to altered drug disposition and abnormal action in affected persons. See, e.g., Eichelbaum, M., Clin. Exp. Pharmacol. Physiol. 23(10-11):983-985 (1996), and Linder, M. W., Clin. Chem. 43(2):254-266 (1997). The clinical outcomes of these variations result in severe toxicity of therapeutic drugs in certain individuals or
15 therapeutic failure of drugs in certain individuals as a result of individual variation in metabolism. Thus, the genotype of the individual can determine the way a therapeutic compound acts on the body or the way the body metabolizes the compound. Further, the activity of drug metabolizing enzymes effects both the intensity and duration of drug action. Thus, the pharmacogenomics of the individual
20 permit the selection of effective compounds and effective dosages of such compounds for prophylactic or therapeutic treatment based on the individual's genotype. Accordingly, in one aspect of the invention, natural variants of the BK channel are used to screen for compounds that are effective against a given allele and are not toxic to the specific patient. Compounds can thus be classed according to
25 their effects against naturally occurring allelic variants. This allows more effective treatment and diagnosis of GEPD.

Test systems for identifying compounds include both cell-free and cell-based systems derived from normal and affected tissue, cell lines and strains, primary

cultures, animal GEPD models, and including transgenic animals.

Naturally-occurring cells can express abnormal levels of *KCNMA1* gene or gene product or variants of *KCNMA1* genes or gene products. Alternatively, these cells can provide recombinant hosts for the expression of desired levels of *KCNMA1* gene or gene product or variants of *KCNMA1* gene or gene product. A cell-free system can be used, for example, when assessing the effective agents on nucleic acid or polypeptide function.

For example, in a cell-free system, competition binding assays are designed to discover compounds that interact with the polypeptide. Thus, a compound is exposed to the polypeptide under conditions that allow the compound to bind or to otherwise interact with the polypeptide. Soluble polypeptide is also added to the mixture. If the test compound interacts with the soluble polypeptide, it decreases the amount of complex formed or activity from the target. This type of assay is particularly useful in cases in which compounds are sought that interact with specific regions of the polypeptide. Thus, the soluble polypeptide that competes with the target region is designed to contain peptide sequences corresponding to the region of interest.

To perform cell-free drug screening assays, it is desirable to immobilize either the protein, or fragment, or its target molecule to facilitate separation of complexes from uncomplexed forms of one or both of the proteins, as well as to accommodate automation of the assay. Techniques for immobilizing proteins on matrices can be used in the drug screening assays. In one embodiment, a fusion protein can be provided which adds a domain that allows the protein to be bound to a matrix. For example, glutathione-S-transferase/BK channel fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, Mo.) or glutathione derivatized microtitre plates, which are then combined with the cell lysates (*e.g.*, ³⁵S-labeled) and the candidate compound, and the mixture incubated under conditions conducive to complex formation (*e.g.*, at physiological conditions

for salt and pH). Following incubation, the beads are washed to remove any unbound label, and the matrix immobilized and radiolabel determined directly, or in the supernatant after the complexes are dissociated. Alternatively, the complexes can be dissociated from the matrix, separated by SDS-PAGE, and the level of BK channel binding protein found in the bead fraction quantified from the gel using standard electrophoretic techniques. For example, either the polypeptide or its target molecule can be immobilized utilizing conjugation of biotin and streptavidin using techniques well known in the art.

Alternatively, antibodies reactive with the protein but which do not interfere with binding of the protein to its target molecule can be derivatized to the wells of the plate, and the protein trapped in the wells by antibody conjugation. Preparations of an BK channel-binding protein and a candidate compound are incubated in the BK channel protein-presenting wells and the amount of complex trapped in the well can be quantitated. Methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies reactive with the BK channel protein target molecule, or which are reactive with the BK channel protein and compete with the target molecule; as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with the target molecule.

Cell-based systems include assay of individual cells or assay of cells in a tissue sample or *in vivo*. Drug screening assays can be cell-based or cell-free systems. Cell-based systems can be native, *i.e.*, cells that normally express the protein, as a biopsy or expanded in cell culture. In one embodiment, however, cell-based assays involve recombinant host cells expressing the protein. *In vivo* test systems include, not only individuals involved in clinical trials, but also animal GEPD models, including transgenic animals. Single cells include recombinant host cells in which desired altered *KCNMA1* gene or gene products have been introduced. These host cells can express abnormally high or low levels of the *KCNMA1* gene or

gene product or mutant versions of the *KCNMA1* gene or gene product. Thus, the recombinant cells can be used as test systems for identifying compounds that have the desired effect on the altered gene or gene product. Mutations can be naturally occurring or constructed for their effect on the course or development of GEPD, for example, determined by the model test systems discussed further below. Similarly, naturally-occurring or designed mutations can be introduced into transgenic animals, which then serve as an *in vivo* test system to identify compounds having a desired effect on *KCNMA1* gene or gene product.

Modulators of *KCNMA1* gene or gene product identified according to these assays can be used to treat GEPD by treating cells that aberrantly express the mutant or variant gene or gene product. These methods of treatment include the steps of administering the modulators of protein activity in a pharmaceutical composition as described herein, to a subject in need of such treatment. The invention thus provides a method for identifying a compound that can be used to treat autosomal GEPD. The method typically includes assaying the ability of the compound to modulate the expression of the *KCNMA1* gene or gene product to identify a compound that can be used to treat the disorder.

The invention is also directed to *KCNMA1* genes or gene products containing alterations that correlate with GEPD. These altered genes or gene products can be isolated and purified or can be created *in situ*, for example, by means of *in situ* gene replacement techniques. In the gene, alterations of this type can be found in any site, transcribed, nontranscribed, coding, and noncoding. Likewise, in the RNA, alterations can be found in both the coding and noncoding regions. In a specific disclosed embodiment, the present invention includes a heterozygous A→G transition identified in exon 10 of the *KCNMA1* gene that corresponds with the nucleotide sequence of SEQ ID NO: 2. In another embodiment, the present includes BK channel α -subunit proteins that have a D434G point mutation in the RCK domain (e.g., SEQ ID NO: 4). The present invention also includes *KCNMA1* gene or

gene products that comprises a fragment, preferably a fragment containing the mutation. The invention thus encompasses primers, both wild type and variant, that are useful in the methods described herein. Similarly, ribozymes and antisense nucleic acids can be derived from variants that correlate with GEPD or can be
5 derived from the wild type and used in the methods described herein.

The genes and gene products are useful in pharmaceutical compositions for diagnosing or modulating the level or expression of *KCNMA1* gene or gene product *in vivo*, as in individual patients treated for GEPD, subjects in clinical trials, animal GEPD models, and transgenic animal GEPD models. Thus, these pharmaceutical
10 compositions are useful for testing and treatment. The *KCNMA1* genes or gene products are also useful for otherwise modulating expression of the gene or gene product in cell-free or cell-based systems *in vitro*. They are further useful in *ex vivo* applications. The *KCNMA1* genes and gene products are also useful for creating model test systems for GEPD, for example, recombinant cells, tissues, and animals.
15 The genes and gene products are also useful in a diagnostic context as comparisons for other naturally-occurring variation in the *KCNMA1* gene or gene product. Accordingly, these reagents can form the basis for a diagnostic kit. Further, specific variants (mutants) are useful for testing compounds that may be effective in the treatment or diagnosis of GEPD. Such mutants can also form the basis of a reagent
20 in a test kit, particularly for introduction into a desired cell type or transgenic animal for drug testing. Accordingly, the invention is also directed to isolated and purified polypeptides and polynucleotides.

The present invention thus also relates to compositions based on *KCNMA1* genes or gene products. Compositions also include nucleic acid primers derived
25 from *KCNMA1* mutants, antisense nucleotides derived from these mutants, and ribozymes based on the mutations, and antibodies specific for the mutants. Compositions further include recombinant cells containing any of the mutants, vectors containing the mutants, cells expressing the mutants, fragments of the

mutants, and antibodies or other binding partners that specifically recognize the mutation. These compositions can all be combined with a pharmaceutically acceptable carrier to create pharmaceutical compositions useful for detecting or modulating the level or expression of *KCNMA1* gene or gene products and thereby diagnosing or treating GEPD.

As used herein, a polypeptide is said to be "isolated" or "purified" when it is substantially free of cellular material when it is isolated from recombinant and non-recombinant cells, or free of chemical precursors or other chemicals when it is chemically synthesized. A polypeptide, however, can be joined to another polypeptide with which it is not normally associated in a cell and still be considered "isolated" or "purified." The *KCNMA1* polypeptides (or proteins) can be purified to homogeneity. It is understood, however, that preparations in which the polypeptide is not purified to homogeneity are useful and considered to contain an isolated form of the polypeptide. The critical feature is that the preparation allows for the desired function of the polypeptide, even in the presence of considerable amounts of other components. Thus, the invention encompasses various degrees of purity.

In one embodiment, the language "substantially free of cellular material" includes preparations of the protein having less than about 30% (by dry weight) other proteins (*i.e.*, contaminating protein), less than about 20% other proteins, less than about 10% other proteins, or less than about 5% other proteins. When the BK channel protein is recombinantly produced, it can also be substantially free of culture medium, *i.e.*, culture medium represents less than about 20%, less than about 10%, or less than about 5% of the volume of the protein preparation.

The language "substantially free of chemical precursors or other chemicals" includes preparations of the polypeptide in which it is separated from chemical precursors or other chemicals that are involved in its synthesis. In one embodiment, the language "substantially free of chemical precursors or other chemicals" includes preparations of the polypeptide having less than about 30% (by dry weight)

chemical precursors or other chemicals, less than about 20% chemical precursors or other chemicals, less than about 10% chemical precursors or other chemicals, or less than about 5% chemical precursors or other chemicals.

5 Variants can be naturally-occurring or can be made by recombinant means or chemical synthesis to provide useful and novel characteristics for the polypeptide. This includes preventing immunogenicity from pharmaceutical formulations by preventing protein aggregation. Useful variations further include alteration of binding characteristics. For example, one embodiment involves a variation at the binding site that results in binding but not release, or slower release, of substrate. A
10 further useful variation at the same sites can result in a higher affinity for substrate. Useful variations also include changes that provide for affinity for another substrate. Another useful variation includes one that allows binding but which reduces cleavage of the substrate.

Amino acids that are essential for function of *KCNMA1* transcription factor
15 can be identified by methods known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham et al., Science 244:1081-1085 (1989)). The latter procedure introduces single alanine mutations at every residue in the molecule. The resulting mutant molecules are then tested for biological activity. Sites that are critical can also be determined by structural analysis such as
20 crystallization, nuclear magnetic resonance or photoaffinity labeling (Smith et al., J. Mol. Biol. 224:899-904 (1992); de Vos et al. Science 255:306-312 (1992)).

The invention also provides antibodies that selectively bind to the BK channel α -subunit protein. An antibody is considered to selectively bind, even if it also binds to other proteins that are not substantially homologous with the BK
25 channel α -subunit protein. These other proteins share homology with a fragment or domain of the protein. This conservation in specific regions gives rise to antibodies that bind to both proteins by virtue of the homologous sequence. In this case, it

would be understood that antibody binding to the BK channel α -subunit protein is still selective.

To generate antibodies, an isolated polypeptide is used as an immunogen to generate antibodies using standard techniques for polyclonal and monoclonal antibody preparation. Either the full-length protein or antigenic peptide fragment can be used. Antibodies are preferably prepared from these regions or from discrete fragments in these regions. However, antibodies can be prepared from any region of the peptide as described herein. A preferred fragment produces an antibody that diminishes or completely prevents substrate-binding. Antibodies can be developed against the entire protein or portions of the protein, for example, the substrate binding domain.

Antibodies can be polyclonal or monoclonal. An intact antibody, or a fragment thereof can be used. Detection can be facilitated by coupling (*i.e.*, physically linking) the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β -galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include ^{125}I , ^{131}I , ^{35}S or ^3H .

An appropriate immunogenic preparation can be derived from native, recombinantly expressed, protein or chemically synthesized peptides. The antibodies can be used to isolate a BK channel α -subunit protein by standard techniques, such as affinity chromatography or immunoprecipitation. The

antibodies can facilitate the purification of the natural protein from cells and recombinantly-produced protein expressed in host cells.

The antibodies are useful to detect the presence of protein in cells or tissues to determine the pattern of expression of the protein among various tissues in an organism. The antibodies can be used to detect the protein *in situ*, *in vitro*, or in a cell lysate or supernatant in order to evaluate the abundance and pattern of expression. The antibodies can be used to assess abnormal tissue distribution or abnormal expression during development. Antibody detection of circulating fragments of the full length of the BK channel α -subunit protein can be used to identify the BK channel α -subunit protein turnover.

Further, the antibodies can be used to assess *KCNMA1* expression in active stages of GEPD or in an individual with a predisposition toward GEPD. The antibodies can also be used to assess normal and aberrant subcellular localization of cells in the various tissues in an organism. Antibodies can be developed against the whole the BK channel α -subunit protein or portions of the BK channel α -subunit protein. The diagnostic uses can be applied, not only in genetic testing, but also in monitoring a treatment modality. Accordingly, where treatment is ultimately aimed at correcting the BK channel α -subunit protein expression level or the presence of aberrant the BK channel α -subunit protein, antibodies directed against the BK channel α -subunit protein or relevant fragments can be used to monitor therapeutic efficacy. The antibodies are also useful for inhibiting the BK channel function. These uses can also be applied in a therapeutic context. Antibodies can be prepared against specific fragments containing sites required for function or against intact BK channel associated with a cell.

An "isolated" *KCNMA1* nucleic acid is one that is separated from other nucleic acid present in the natural source of the *KCNMA1* nucleic acid. Preferably, an "isolated" nucleic acid is free of sequences which naturally flank the nucleic acid (*i.e.*, sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA

of the organism from which the nucleic acid is derived. However, there can be some flanking nucleotide sequences, for example up to about 5KB. The important point is that the nucleic acid is isolated from flanking sequences such that it can be subjected to the specific manipulations described herein such as recombinant expression, preparation of probes and primers, and other uses specific to the nucleic acid sequences.

Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or chemical precursors or other chemicals when chemically synthesized. However, the nucleic acid molecule can be fused to other coding or regulatory sequences and still be considered isolated.

For example, recombinant DNA molecules contained in a vector are considered isolated. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or substantially) DNA molecules in solution. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of the isolated DNA molecules of the present invention. Isolated nucleic acid molecules according to the present invention further include such molecules produced synthetically.

The *KCNMA1* polynucleotides can encode the mature protein plus additional amino or carboxyl-terminal amino acids, or amino acids interior to the mature polypeptide (when the mature form has more than one polypeptide chain, for instance). Such sequences may play a role in processing of a protein from precursor to a mature form, facilitate protein trafficking, prolong or shorten protein half-life or facilitate manipulation of a protein for assay or production, among other things. As generally is the case *in situ*, the additional amino acids may be processed away from the mature protein by cellular enzymes.

The *KCNMA1* polynucleotides include, but are not limited to, the sequence encoding the mature polypeptide alone, the sequence encoding the mature

polypeptide and additional coding sequences, such as a leader or secretory sequence (e.g., a pre-pro or pro-protein sequence), the sequence encoding the mature polypeptide, with or without the additional coding sequences, plus additional non-coding sequences, for example introns and non-coding 5' and 3' sequences such as transcribed but non-translated sequences that play a role in transcription, mRNA processing (including splicing and polyadenylation signals), ribosome binding and stability of mRNA. In addition, the polynucleotide may be fused to a marker sequence encoding, for example, a peptide that facilitates purification.

Polynucleotides can be in the form of RNA, such as mRNA, or in the form DNA, including cDNA and genomic DNA obtained by cloning or produced by chemical synthetic techniques or by a combination thereof. The nucleic acid, especially DNA, can be double-stranded or single-stranded. Single-stranded nucleic acid can be the coding strand (sense strand) or the non-coding strand (anti-sense strand).

The invention also provides *KCNMA1* nucleic acid molecules encoding the variant polypeptides described herein (e.g., SEQ ID NO: 2). Such polynucleotides may be naturally-occurring, such as allelic variants (same locus), homologs (different locus), and orthologs (different organism), or may be constructed by recombinant DNA methods or by chemical synthesis. Such non-naturally occurring variants may be made by mutagenesis techniques, including those applied to polynucleotides, cells, or organisms. Accordingly, as discussed above, the variants can contain nucleotide substitutions, deletions, inversions and insertions.

Variation can occur in either or both the coding and non-coding regions. The variations can produce both conservative and non-conservative amino acid substitutions. Furthermore, the invention provides polynucleotides that comprise a fragment of the full length *KCNMA1* polynucleotides. The fragment can be single or double stranded and can comprise DNA or RNA. The fragment can be derived from either the coding or the non-coding sequence.

The invention also provides *KCNMA1* nucleic acid fragments that encode epitope bearing regions of the BK channel α -subunit protein described herein. The invention also provides vectors containing the *KCNMA1* polynucleotides. The term "vector" refers to a vehicle, preferably a nucleic acid molecule, that can transport the *KCNMA1* polynucleotides. When the vector is a nucleic acid molecule, the *KCNMA1* polynucleotides are covalently linked to the vector nucleic acid. With this aspect of the invention, the vector includes a plasmid, single or double stranded phage, a single or double stranded RNA or DNA viral vector, or artificial chromosome, such as a BAC, PAC, YAC, OR MAC.

A vector can be maintained in the host cell as an extrachromosomal element where it replicates and produces additional copies of the *KCNMA1* polynucleotides. Alternatively, the vector may integrate into the host cell genome and produce additional copies of the *KCNMA1* polynucleotides when the host cell replicates. The invention provides vectors for the maintenance (cloning vectors) or vectors for expression (expression vectors) of the *KCNMA1* polynucleotides. The vectors can function in prokaryotic or eukaryotic cells or in both (shuttle vectors).

Expression vectors contain cis-acting regulatory regions that are operably linked in the vector to the *KCNMA1* polynucleotides such that transcription of the polynucleotides is allowed in a host cell. The polynucleotides can be introduced into the host cell with a separate polynucleotide capable of affecting transcription. Thus, the second polynucleotide may provide a trans-acting factor interacting with the cis-regulatory control region to allow transcription of the *KCNMA1* polynucleotides from the vector. Alternatively, a trans-acting factor may be supplied by the host cell. Finally, a transacting factor can be produced from the vector itself.

It is understood, however, that in some embodiments, transcription and/or translation of the *KCNMA1* polynucleotides can occur in a cell free system. The regulatory sequence to which the polynucleotides described herein can be operably linked include promoters for directing mRNA transcription. These include, but are

not limited to, the left promoter from bacteriophage λ , the lac, TRP, and TAC promoters from *E. coli*, the early and late promoters from SV40, the CMV immediate early promoter, the adenovirus early and late promoters, and retrovirus long-terminal repeats.

5 In addition to control regions that promote transcription, expression vectors may also include regions that modulate transcription, such as repressor binding sites and enhancers. Examples include the SV40 enhancer, the cytomegalovirus immediate early enhancer, polyoma enhancer, adenovirus enhancers, and retrovirus LTR enhancers.

10 In addition to containing sites for transcription initiation and control, expression vectors can also contain sequences necessary for transcription termination and, in the transcribed region a ribosome binding site for translation. Other regulatory control elements for expression include initiation and termination codons as well as polyadenylation signals. The person of ordinary skill in the art
15 would be aware of the numerous regulatory sequences that are useful in expression vectors. Such regulatory sequences are described, for example, in Sambrook et al., *Molecular Cloning: A Laboratory Manual*. 2nd. ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., (1989).

 A variety of expression vectors can be used to express a *KCNMA1*
20 polynucleotide. Such vectors include chromosomal, episomal, and virus-derived vectors, for example vectors derived from bacterial plasmids, from bacteriophage, from yeast episomes, from yeast chromosomal elements, including yeast artificial chromosomes, from viruses such as baculoviruses, papovaviruses such as SV40, Vaccinia viruses, adenoviruses, poxviruses, pseudorabies viruses, and retroviruses.
25 Vectors may also be derived from combinations of these sources such as those derived from plasmid and bacteriophage genetic elements, e.g. cosmids and phagemids. Appropriate cloning and expression vectors for prokaryotic and eukaryotic hosts are described in Sambrook et al., *Molecular Cloning: A Laboratory*

Manual. 2nd. ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., (1989).

The regulatory sequence may provide constitutive expression in one or more host cells (*i.e.* tissue specific) or may provide for inducible expression in one or more cell types such as by temperature, nutrient additive, or exogenous factor such as a hormone or other ligand. A variety of vectors providing for constitutive and inducible expression in prokaryotic and eukaryotic hosts are well known to those of ordinary skill in the art.

The *KCNMA1* polynucleotides can be inserted into the vector nucleic acid by well-known methodology. Generally, the DNA sequence that will ultimately be expressed is joined to an expression vector by cleaving the DNA sequence and the expression vector with one or more restriction enzymes and then ligating the fragments together. Procedures for restriction enzyme digestion and ligation are well known to those of ordinary skill in the art.

The vector containing the appropriate polynucleotide can be introduced into an appropriate host cell for propagation or expression using well-known techniques. Bacterial cells include, but are not limited to, *E. coli*, *Streptomyces*, and *Salmonella typhimurium*. Eukaryotic cells include, but are not limited to, yeast, insect cells such as *Drosophila*, animal cells such as COS and CHO cells, and plant cells.

As described herein, it may be desirable to express the polypeptide as a fusion protein. Accordingly, the invention provides fusion vectors that allow for the production of the BK channel polypeptides. Fusion vectors can increase the expression of a recombinant protein, increase the solubility of the recombinant protein, and aid in the purification of the protein by acting for example as a ligand for affinity purification. A proteolytic cleavage site may be introduced at the junction of the fusion moiety so that the desired polypeptide can ultimately be separated from the fusion moiety. Proteolytic enzymes include, but are not limited to, factor Xa, thrombin, and enterokinase. Typical fusion expression vectors include

pGEX (Smith et al., Gene 67:31-40 (1988)), pMAL (New England Biolabs, Beverly, Mass.) and pRIT5 (Pharmacia, Piscataway, N.J.) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein. Examples of suitable inducible non-fusion E. coli expression vectors include pTrc (Amann et al., Gene 69:301-315 (1988)) and pET 11d (Studier et al., Gene Expression Technology: Methods in Enzymology 185:60-89 (1990)).

Recombinant protein expression can be maximized in a host bacteria by providing a genetic background wherein the host cell has an impaired capacity to proteolytically cleave the recombinant protein. (Gottesman, S., Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, Calif. (1990) 119-128). Alternatively, the sequence of the polynucleotide of interest can be altered to provide preferential codon usage for a specific host cell, for example E. coli. (Wada et al., Nucleic Acids Res. 20:2111-2118 (1992)).

In certain embodiments of the invention, the polynucleotides described herein are expressed in mammalian cells using mammalian expression vectors. Examples of mammalian expression vectors include pCDM8 (Seed, B. Nature 329:840(1987)) and pMT2PC (Kaufman et al, EMBO J. 6:187-195 (1987)).

The expression vectors listed herein are provided by way of example only of the well-known vectors available to those of ordinary skill in the art that would be useful to express the *KCNMA1* polynucleotides. The person of ordinary skill in the art would be aware of other vectors suitable for maintenance propagation or expression of the polynucleotides described herein. These are found for example in Sambrook, J., Fritsh, E. F., and Maniatis, T. Molecular Cloning: A Laboratory Manual 2nd, ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989.

The invention also encompasses vectors in which the nucleic acid sequences described herein are cloned into the vector in reverse orientation, but operably

linked to a regulatory sequence that permits transcription of antisense RNA. Thus, an antisense transcript can be produced to all, or to a portion, of the polynucleotide sequences described herein, including both coding and noncoding regions.

Expression of this antisense RNA is subject to each of the parameters described
5 above in relation to expression of the sense RNA (regulatory sequences, constitutive or inducible expression, tissue-specific expression).

The invention also relates to recombinant host cells containing the vectors described herein. Host cells therefore include prokaryotic cells, lower eukaryotic cells such as yeast, other eukaryotic cells such as insect cells, and higher eukaryotic
10 cells such as mammalian cells.

The recombinant host cells are prepared by introducing the vector constructs described herein into the cells by techniques readily available to the person of ordinary skill in the art. These include, but are not limited to, calcium phosphate transfection, DEAE-dextran-mediated transfection, cationic lipid-mediated
15 transfection, electroporation, transduction, infection, lipofection, and other techniques such as those found in Sambrook, et al. (Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989).

Host cells can contain more than one vector. Thus, different nucleotide
20 sequences can be introduced on different vectors of the same cell. Similarly, the *KCNMA1* polynucleotides can be introduced either alone or with other polynucleotides that are not related to the *KCNMA1* polynucleotides such as those providing trans-acting factors for expression vectors. When more than one vector is introduced into a cell, the vectors can be introduced independently, co-introduced or
25 joined to the *KCNMA1* polynucleotide vector.

In the case of bacteriophage and viral vectors, these can be introduced into cells as packaged or encapsulated virus by standard procedures for infection and transduction. Viral vectors can be replication-competent or replication-defective. In

the case in which viral replication is defective, replication will occur in host cells providing functions that complement the defects.

Vectors generally include selectable markers that enable the selection of the subpopulation of cells that contain the recombinant vector constructs. The marker
5 can be contained in the same vector that contains the polynucleotides described herein or may be on a separate vector. Markers include tetracycline or ampicillin-resistance genes for prokaryotic host cells and dihydrofolate reductase or neomycin resistance for eukaryotic host cells. However, any marker that provides selection for a phenotypic trait will be effective. While the mature proteins can be
10 produced in bacteria, yeast, mammalian cells, and other cells under the control of the appropriate regulatory sequences, cell free transcription and translation systems can also be used to produce these proteins using RNA derived from the DNA constructs described herein.

It is also understood that depending upon the host cell in recombinant
15 production of the polypeptides described herein, the polypeptides can have various glycosylation patterns, depending upon the cell, or maybe non-glycosylated as when produced in bacteria. In addition, the polypeptides may include an initial modified methionine in some cases as a result of a host-mediated process.

The host cells expressing the polypeptides described herein, and particularly
20 recombinant host cells, have a variety of uses. First, the cells are useful for producing the BK channel α -subunit protein or polypeptides that can be further purified to produce desired amounts of the BK channel α -subunit protein or fragments. Thus, host cells containing expression vectors are useful for polypeptide production. Host cells are also useful for conducting cell based assays involving the
25 *KCNMA1* or *KCNMA1* fragments. Thus, a recombinant host cell expressing a native BK channel α -subunit protein is useful to assay for compounds that stimulate or inhibit the BK channel α -subunit protein function.

Host cells are also useful for identifying *KCNMA1* mutants in which these functions are affected. If the mutants naturally occur, host cells containing the mutations are useful to assay compounds that have a desired effect on the mutant *KCNMA1* (for example, stimulating or inhibiting function) which may not be indicated by their effect on the native *KCNMA1*.

Recombinant host cells are also useful for expressing the chimeric polypeptides described herein to assess compounds that activate or suppress activation by means of a heterologous amino terminal extracellular domain (or other binding region). Alternatively, a heterologous region spanning the entire transmembrane domain (or parts thereof) can be used to assess the effect of a desired amino terminal extracellular domain (or other binding region) on any given host cell. In this embodiment, a region spanning the entire transmembrane domain (or parts thereof) compatible with the specific host cell is used to make the chimeric vector. Alternatively, a heterologous carboxy terminal intracellular, *e.g.*, signal transduction, domain can be introduced into the host cell.

Further, mutant BK channel α -subunit proteins can be designed in which one or more of the various functions is engineered to be increased or decreased used to augment or replace the BK channel α -subunit protein in an individual. Thus, host cells can provide a therapeutic benefit by replacing an aberrant the BK channel α -subunit protein or providing an aberrant BK channel α -subunit protein that provides a therapeutic result. In one embodiment, the cells provide BK channel α -subunit protein that is abnormally active.

Homologously recombinant host cells can also be produced that allow the *in situ* alteration of endogenous *KCNMA1* polynucleotide sequences in a host cell genome. This technology is more fully described in U.S. Pat. No. 5,641,670. Briefly, specific polynucleotide sequences corresponding to the *KCNMA1* polynucleotides or sequences proximal or distal to a *KCNMA1* gene are allowed to integrate into a host cell genome by homologous recombination where expression of the gene can be

affected. In one embodiment, regulatory sequences are introduced that either increase or decrease expression of an endogenous sequence. Accordingly, a the BK channel α -subunit protein can be produced in a cell not normally producing it, or increased expression of the BK channel α -subunit protein can result in a cell normally producing the protein at a specific level.

The genetically engineered host cells can be used to produce non-human transgenic animals. A transgenic animal is preferably a mammal, for example a rodent, such as a rat or mouse, in which one or more of the cells of the animal include a transgene. A transgene is exogenous DNA which is integrated into the genome of a cell from which a transgenic animal develops and which remains in the genome of the mature animal in one or more cell types or tissues of the transgenic animal. These animals are useful for studying the function of the BK channel α -subunit protein and identifying and evaluating modulators of the BK channel α -subunit protein activity. Other examples of transgenic animals include non-human primates, sheep, dogs, cows, goats, chickens, and amphibians.

In one embodiment, a host cell is a fertilized oocyte or an embryonic stem cell into which *KCNMA1* polynucleotide sequences have been introduced. A transgenic animal can be produced by introducing nucleic acid into the male pronuclei of a fertilized oocyte, *e.g.*, by microinjection, retroviral infection, and allowing the oocyte to develop in a pseudopregnant female foster animal. Any of the *KCNMA1* nucleotide sequences described herein, especially the altered sequences, can be introduced as a transgene into the genome of a non-human animal, such as a mouse.

Any of the regulatory or other sequences useful in expression vectors can form part of the transgenic sequence. This includes intronic sequences and polyadenylation signals, if not already included. A tissue-specific regulatory sequence(s) can be operably linked to the transgene to direct expression of the BK channel α -subunit protein to particular cells.

Methods for generating transgenic animals via embryo manipulation and microinjection, particularly animals such as mice, have become conventional in the art and are described, for example, in U.S. Pat. Nos. 4,736,866 and 4,870,009, both by Leder et al., U.S. Pat. No. 4,873,191 by Wagner et al. and in Hogan, B.,
5 Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986). Similar methods are used for production of other transgenic animals. A transgenic founder animal can be identified based upon the presence of the transgene in its genome and/or expression of transgenic mRNA in tissues or cells of the animals. A transgenic founder animal can then be used to
10 breed additional animals carrying the transgene. Moreover, transgenic animals carrying a transgene can further be bred to other transgenic animals carrying other transgenes. A transgenic animal also includes animals in which the entire animal or tissues in the animal have been produced using the homologously recombinant host cells described herein.

15 Clones of the non-human transgenic animals described herein can also be produced according to the methods described in Wilmut, I. et al. Nature 385:810-813 (1997) and PCT International Publication Nos. WO 97/07668 and WO 97/07669. In brief, a cell, *e.g.*, a somatic cell, from the transgenic animal can be isolated and induced to exit the growth cycle and enter G0 phase. The quiescent cell
20 can then be fused, *e.g.*, through the use of electrical pulses, to an enucleated oocyte from an animal of the same species from which the quiescent cell is isolated. The reconstructed oocyte is then cultured such that it develops to morula or blastocyst and then transferred to a pseudopregnant female foster animal. The offspring born of this female foster animal will be a clone of the animal from which the cell, *e.g.*,
25 the somatic cell, is isolated.

Transgenic animals containing recombinant cells that express the polypeptides described herein are useful to conduct the assays described herein in an *in vivo* context. Accordingly, the various physiological factors that are present *in*

vivo and that could effect ligand binding, *KCNMA1* activation, and signal transduction, may not be evident from *in vitro* cell free or cell based assays.

Accordingly, it is useful to provide non-human transgenic animals to assay *in vivo* *KCNMA1* function, the effect of specific mutant *KCNMA1*s on *KCNMA1* function, and the effect of chimeric *KCMAN1*s. It is also possible to assess the effect of null mutations, that is mutations that substantially or completely eliminate one or more *KCNMA1* functions.

The *KCNMA1* nucleic acid molecules, protein (particularly fragments, such as the domains that interact with other cellular components), modulators of the nucleic acid and protein, and especially binding partners, and antibodies (also referred to herein as "active compounds") can be incorporated into pharmaceutical compositions suitable for administration to a subject, *e.g.*, a human. Such compositions typically comprise the nucleic acid molecule, protein, modulator, or antibody and a pharmaceutically acceptable carrier.

As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, such media can be used in the compositions of the invention. Supplementary active compounds can also be incorporated into the compositions. A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, (*e.g.*, intravenous, intradermal, subcutaneous), oral (*e.g.*, inhalation), transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils,

polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL (BASF, Parsippany, N.J.) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by

including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound (*e.g.*, a BK channel α -subunit protein or anti- BK channel α -subunit antibody) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For oral administration, the agent can be contained in enteric forms to survive the stomach or further coated or mixed to be released in a particular region of the GI tract by known methods. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent

such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable
5 propellant (*e.g.*, a gas such as carbon dioxide) or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile
10 salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art. The compounds can also be prepared in the form of suppositories (*e.g.*, with conventional suppository bases such as cocoa butter
15 and other glycerides) or retention enemas for rectal delivery.

In one aspect of the invention, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as
20 ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral
25 antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Pat. No. 4,522,811.

It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. "Dosage unit form" as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

The nucleic acid molecules of the invention can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (U.S. Pat. No. 5,328,470) or by stereotactic injection (see *e.g.*, Chen et al., PNAS 91:3054-3057 (1994)). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells (*e.g.*, retroviral vectors) the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration. This invention may be embodied in many different forms and should not be construed as limited to the embodiments set forth herein; rather, these embodiments are provided so that this disclosure will fully convey the invention to those skilled in the art. Many modifications and other embodiments of the invention will come to mind in one skilled in the art to which this invention pertains having the benefit of the teachings

presented in the foregoing description. Although specific terms are employed, they are used as in the art unless otherwise indicated.

The following examples are included to demonstrate various aspects of the invention. Those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific aspects which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLES

We studied a large, five-generation, 31-member family with co-existent generalized epilepsy and PD (GEPD). Sixteen affected individuals developed epileptic seizures (n=4), non-kinesigenic PD (n=7), or both (n=5) (Fig. 1a). The detailed clinical features of 13 affected individuals, who participated in subsequent genetic studies, are summarized in Table 1. Episodes of dyskinesia in the family were not associated with any epileptic EEG changes, indicating the co-existence of two distinct disorders, dyskinesia and epilepsy, in the family. PD can be broadly classified into two main groups: paroxysmal kinesigenic dyskinesia (PKD) if the attacks are induced by sudden movement and paroxysmal non-kinesigenic dyskinesia (PNKD) if they are not. PD in the family under study is PNKD. Pedigree analysis suggested an autosomal dominant pattern of inheritance. We carried out a genome-wide linkage scan for the family with 382 microsatellite markers that span human chromosomes 1-22 by an average of every 10 cM. Markers *D10S580* and *D10S1730* on chromosome 10q22 showed significant linkage to GEPD with a LOD score of 3.68 and 3.73 at a recombination fraction θ of 0, respectively. The chromosomal 10q22 region represents the only region with a LOD score of ≥ 2.0 in the whole genome. Fine mapping and haplotype analysis using eight additional markers near *D10S580* and *D10S1730* defined the disease gene within an 8.4 cM region on chromosome 10q22 flanked by markers *D10S1694* and *D10S201* (Fig. 1a, b). Individual III-4 showed an obligate recombination between markers *D10S1694*

and *D10S580* (Fig. 1a), defining *D10S1694* as the centromeric marker for the disease locus. Individual IV-4 with a recombination between *D10S201* and *D10S1730* (Fig. 1a) placed *D10S201* as the telomeric flanking marker for the disease locus.

5 The 10q22 GEPD locus contains 40 genes, including 33 known genes and 7 hypothetical genes. We hypothesized that mutations in ion channel genes cause GEPD, and identified two ion channel genes at the disease locus: *VDAC2* encoding voltage-dependent anion channel 2 and *KCNMA1* coding for the pore-forming α -subunit of the large conductance calcium-activated potassium (BK or Maxi-K) channel. Both genes were analyzed for mutations by direct DNA sequence analysis. We did not find any mutation in *VDAC2*. Interestingly, a heterozygous A→G transition was identified in exon 10 of the *KCNMA1* gene in the proband (IV-8) of the family (Fig. 2a, reverse sequence). The A→G transition results in the substitution of a negatively charged aspartic acid residue for a neutral glycine residue (D434G) in the RCK domain (regulator of conductance for K⁺) (Fig. 2a, b). Amino acid residue D434 is conserved among *KCNMA1* channels from *C. elegans* to the human (Fig. 2c). DNA sequence analysis revealed the presence of mutation D434G in all 13 affected individuals that were genotyped and the absence in 5 normal individuals in the family (data not shown). This result was confirmed by restriction fragment length polymorphism (RFLP) analysis as the mutation creates a *Tsp45I* restriction site. As shown in Fig. 2d, mutation D434G co-segregates with the affected individuals in the family, but not with the unaffected individuals. Furthermore, the mutation was not detected in 400 unrelated healthy controls. These results strongly suggest that the D434G mutation of *KCNMA1* is responsible for GEPD in this large family.

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The BK channel is activated by both membrane depolarization and a rise in cytosolic Ca²⁺ (5). The pore-forming α -subunit contains seven transmembrane domains (S0-S6) at the N terminus and an extensive C terminus with four

hydrophobic segments (S7-S10) and the Ca^{2+} bowl (Fig. 2b). Between S6 and S8 is the RCK domain, which may contain binding sites for a variety of regulatory ligands, including Ca^{2+} and Mg^{2+} (Fig. 2b) (6-9). As mutation D434G is located in the RCK domain, we hypothesized that the mutation may cause abnormal calcium affinity of the BK channel. We expressed wild type and mutant D434G BK channels in both *Xenopus* oocytes and mammalian Chinese hamster ovary (CHO) cells, and current-voltage families were recorded. In oocytes expressing D434G channels, at the calcium concentration of 2 μM , there was more current induced at the same membrane potential as compared to oocytes expressing the WT BK channel, and the voltage-dependence of steady-state activation (G-V relation) was shifted more than 57 mV towards more negative potentials, with little change in the slope of the curve (Fig. 3a, b). At the calcium concentration of 0.1 μM , the G-V relation was shifted ~26 mV towards more negative potentials in oocytes expressing mutant D434G channels (Fig. 3b). These results indicate an increased voltage and calcium dependent activation of the mutant BK channel. Corresponding to the shifts in voltage dependence of activation, the D434G currents activated faster than the WT BK currents in response to a depolarizing voltage pulse (Fig. 3 a, c). These results suggest that during an action potential in neurons, in response to depolarization and Ca^{2+} entry through voltage dependent Ca^{2+} channels, more mutant BK channels open, causing a rapid repolarization of the action potential.

To better define the mechanism of the increase in macroscopic currents, single channel recordings were made from BK channels expressed in CHO cells. Both the WT channel and the mutant channel were activated by an increase in voltage and/or intracellular $[\text{Ca}^{2+}]$. However, at a given voltage and $[\text{Ca}^{2+}]$, the mutant channel spent significantly more time in the open state (Fig. 4a). Similar to measurements of macroscopic currents, at a given $[\text{Ca}^{2+}]$ the mutant channel was activated at lower voltages (Fig. 4b). There was no difference in the Boltzmann's slope factor for single channels (WT - 9.7 ± 4.1 ; D434G - 8.8 ± 3.7 - $\pm$; mean $\pm$ SD;

n=9 WT, n=24 D434G; $p>0.05$), and the difference in voltage sensitivity was smaller at saturating levels of Ca^{2+} . These findings point to a 3-5 fold increase in Ca^{2+} sensitivity (Fig. 4c, d), rather than a primary effect on the voltage sensor, which is consistent with the role of the RCK domain as a high affinity site for Ca^{2+} binding.

5 These results are interesting in light of the fact that there are mutations of the α subunit that decrease Ca^{2+} sensitivity of BK channels, but there have been none reported that increase Ca^{2+} sensitivity. There are other mechanisms by which Ca^{2+} sensitivity can be increased, such as by association of the α subunit with the β subunit, and in fact a mutation of the $\beta 1$ subunit (a predominant isoform in smooth
10 muscle) has recently been identified that further increases Ca^{2+} sensitivity. However, the enhancement of Ca^{2+} sensitivity reported here by a change intrinsic to the α subunit is unique. Consistent with the location of the mutation remote from the pore region, there was no change in single channel conductance (WT - 185 ± 16 pS; D434G - 180 ± 20 pS; $p>0.05$; $n=11$; mean $\pm$ SD). Similar results on single channel
15 properties were obtained with oocyte recordings (data not shown).

An increase in calcium sensitivity of the BK channel can lead to greater macroscopic potassium conductance under physiological conditions. Thus, the D434G mutation leads to gain-of-function of the α -subunit. There may be a number of reasons why gain-of-function of the BK channel can lead to an increase in brain
20 excitability, causing generalized epilepsy when the thalamus or thalamo-cortical circuits are involved and paroxysmal dyskinesia when the basal ganglia is involved. We believe that the most likely mechanism relates to a more rapid repolarization of action potentials by the mutant D434G channels. Enhancing this repolarization would enable faster repriming (removal of inactivation) of sodium channels, and
25 thus allow neurons to fire at a higher frequency. There are other possible explanations. For example, enhancing some inhibitory currents can switch neurons within a circuit into a bursting mode, as can occur with absence seizures that depend upon activation of inhibitory GABA_B receptors within the thalamus. Likewise,

gain-of-function of BK channels could lead to greater hyperpolarization and activate the hyperpolarization-activated cation current, I_h , resulting in generation of secondary depolarizations. An alternative explanation is that if BK channels are present in GABAergic neurons, an increase in inhibition of these neurons could lead to disinhibition of a neuronal network.

Ethanol can directly activate the BK channel *in vivo* in *C. elegans*. This finding may explain the observation that alcohol triggers dyskinesias in certain individuals in the family reported here. The gain-of-function mutation D434G may have a synergistic effect with ethanol to trigger the onset of the symptoms.

Recently, knockout mice deficient in the BK channel $\beta 4$ subunit were created and characterized. The $\beta 4$ subunit is a neuron-specific inhibitory subunit for the BK current. The knockout mice for the BK $\beta 4$ subunit increased the BK current, and displayed spontaneous non-convulsive seizures (epilepsy). The seizures disappeared with treatment of paxilline, a specific blocker for BK channels. These results in animals provide strong and independent validation to our conclusion that the gain-of-function of the BK channel causes GEPD.

The *in vivo* physiological roles of *KCNMA1* remain intriguing. Mice deficient in *KCNMA1* have been created, however, the phenotype of these mice suggests modest roles of the BK channel in normal brain function even though its expression is widespread in the central nervous system. Homozygous $BK^{-/-}$ mice displayed abnormal conditioned eye-blink reflex, and abnormal locomotion and motor coordination. These mice also developed high-frequency hearing loss at ≥ 8 weeks of age. The human patients with *KCNMA1* mutation D434G did not show any of these phenotypes reported for the knockout mice. The major reason for the phenotypic difference between the knockout mice and human patients reported here may be that the missense mutation identified here is a gain-of-function mutation compared to the absence of BK channels in mice. Our results provide a clue to the physiological role of the BK potassium channel in the central nervous system, in that

increased BK channel activity can causes epilepsy and dyskinesia, and are the first time that a mutation in the BK channel α -subunit has been shown to cause human neurological disease.

In summary, this study identified a novel genetic locus for GEPD on chromosome 10q22, and establishes that mutations in the BK channel cause GEPD. These results provide a molecular basis for this syndrome, and indicate that a mutation in a single ion channel can lead to simultaneous development of both generalized epilepsy and paroxysmal dyskinesia. Our study also suggests the use of BK channel blocking agents as a potential therapy for epilepsy and paroxysmal dyskinesia. Further studies into the molecular mechanisms of this genetic defect may lead to new targets for therapy.

Table 1

Clinical features of 13 affected individuals in the GEPD family QW1378. Affected family members were diagnosed with either paroxysmal dyskinesia (PD), epilepsy (E), or both. SWC gen, generalized spike wave complexes typical of idiopathic generalized epilepsy; GTC, generalized tonic-clonic seizures.

ID	Age of Epilepsy Onset	Seizure Type	EEG	Age of PD Onset	Diagnosis (E, PD)
II-02	--	--	--	13-15 years	PD
II-03	6 years	possible absence	normal, as adult	6 years	PD, possible E
III-02	8-9 years	possible absence	--	N/A	E
III-04	--	--	--	4-5 years	PD
III-05	--	--	normal, as adult	7 years	PD
III-07	--	--	--	4-5 years	PD
III-09	--	--	--	3-4 years	PD
IV-01	<6 months	absence, rare GTC	SWC gen	<6 months	E + PD
IV-02	3 years	absence, rare GTC	SWC gen	<6 months	E + PD
IV-03	--	--	normal	4-5 years	PD
IV-04	--	--	normal	4-5 years	PD
IV-06	5-6 years	possible absence	normal	5-6 years	PD, possible E
IV-08	2 years	absence	SWC gen	2 years	E + PD

Materials and Methods

Human subjects

This study was approved by the Cleveland Clinic Institutional Review Board on Human Subjects. Informed consent was obtained from all participants or their guardians. The patients and family members were identified and clinically characterized at the Department of Neurology of the Cleveland Clinic Foundation. The family under study is of mixed European descent, and was referred to this study from the adult epilepsy clinic due to the diagnosis of epilepsy in multiple family members. A detailed pedigree was constructed. Clinical information was obtained through semistructured interviews in person and over the phone, conducted by a neurologist with specialty training in epilepsy and clinical neurophysiology. Seizure histories were corroborated by questioning eyewitnesses where possible. Records of interictal EEG and video-EEG were obtained when applicable (Fig. 5). Epilepsy was defined as two or more unprovoked seizures. Seizure types were classified according to the International Classification of Epileptic Seizures (1). Epilepsy syndromes were classified according to the International Classification of Epilepsies and Epileptic Syndromes (2). The proband (IV-8, Fig. 1a) was interviewed at 21 years of age. She had normal birth and early development. At two years of age she developed episodes of involuntary mouth movement and hand stiffness, lasting 10 seconds to two minutes, with preserved consciousness. These occurred weekly, were more common with fatigue, and were not triggered by sudden movement. At approximately the same age she developed separate episodes of loss of awareness, with vacant staring and unresponsiveness, characteristic of typical absence seizures. There was no aura, and these occurred monthly. Routine electroencephalogram (EEG) showed generalized spike-wave-complexes. Her paternal first cousin (IV-1) had episodes of vacant staring and episodes of paroxysmal oral dyskinesias without loss of awareness. She was evaluated with inpatient continuous video-EEG at two years of age. Her interictal EEG showed generalized spike-wave complexes.

Episodes of vacant staring and eyelid fluttering were associated with bursts of generalized spike-wave complexes, confirming epilepsy. Episodes of oral dyskinesias were not associated with any EEG change, confirming their non-epileptic nature. At five years of age she developed generalized tonic-clonic seizures. Epileptic seizures, in those other family members affected with epilepsy, were typically absence seizures, with generalized tonic-clonic seizures in one other individual (IV-2). Paroxysmal dyskinesias, in those affected, were often described as involuntary dystonic or choreiform movements of the mouth, tongue, and extremities, non-kinesigenic but induced by alcohol, fatigue, and stress. These had onset in childhood and showed a gradual decrease in frequency with age, but persisted into the fourth decade in some individuals.

Genotyping and linkage analysis

Human genomic DNA was prepared from whole blood with the DNA Isolation Kit for Mammalian Blood (Roche Diagnostic Co).

Genome-wide genotyping was carried out using 382 polymorphic, fluorescently labeled microsatellite markers on chromosomes 1-22 (ABI PRISM Linkage Mapping Set-MD10). Additional markers were identified at the Genethon database, and used for fine mapping and haplotype analysis. Markers were genotyped using an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Allele-calling was carried out by GeneScan and GeneMapper 2 software programs (Applied Biosystems, Foster City, CA). Linkage analysis and two-point LOD score calculation were performed using the Linkage Package 5.2 assuming autosomal dominant inheritance, penetrance of 99%, a phenocopy rate of 0%, gene frequency of 1/10,000, and allele frequency of 1/n where n equals the number of alleles observed.

Mutational analysis

*The genomic structure of the *KCNMA1* gene was determined by comparing the 3,537 bp cDNA sequence (GenBank accession number NM_002247 SEQ ID

NO: 1) to its genomic sequence, and was found to contain 27 exons (Table 2).

Polymerase chain reaction (PCR) primers were then designed based on intronic sequences to amplify the all 27 coding exons (Table 3). PCR products were purified from agarose gels using the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA) and sequenced with both forward and reverse primers by an ABI3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Restriction fragment length polymorphism (RFLP) analysis was used to confirm the D434G mutation and to test the presence/absence of the mutation in other family members and 400 normal controls. The 201bp PCR fragment containing exon 10 where the D434G mutation is located was digested by incubation with restriction enzyme *Tsp45I*. The digested product was separated by 2% agarose gels, and analyzed.

Cloning and mutagenesis

The human *KCNMA1* cDNA was cloned into plasmid pcDNA3, resulting in an expression construct for the BK channel. The D434G mutation was introduced into the *KCNMA1-pcDNA3* construct by PCR-based site-directed mutagenesis, and confirmed by sequencing of the full *KCNMA1* insert.

To create the expression constructs for *Xenopus* oocyte expression, we subcloned the full-length wild type and mutant *KCNMA1* cDNA into the pSP64 Poly(A) vector (*KCNMA1-pSP64*) using restriction enzymes Hind III and XbaI.

Electrophysiological characterization of human BK channels in *Xenopus* oocytes

KCNMA1-pSP64 DNA was digested with EcoR I, and cRNA was prepared using the In Vitro Transcription kit with SP6 polymerase. 5 ng of cRNA was injected into each *Xenopus laevis* oocyte 2-6 days before recording. Macroscopic currents were recorded from inside-out patches formed with borosilicate pipettes of 0.9~1.8 megohm resistance. Data were acquired using an Axopatch 200-B patch clamp amplifier (Axon Instruments) and Pulse acquisition software (HEKA

Electronik). Records were digitized at 20- μ s intervals and low pass filtered at 10 KHz with the Axopatch's 4 pole Bessel filter. The pipette solution contained (mM): 140 K-Methanesulfonic Acid, 20 Hepes, 2 KCl, 2 MgCl₂, pH 7.20. The basal internal solution contained (mM): 140 K-Methanesulfonic Acid, 20 Hepes, 2 KCl, 1 EGTA, pH 7.20. CaCl₂ was added to internal solutions to give the appropriate free [Ca²⁺]_i. All recordings were obtained at room temperature (22-24 °C).

Electrophysiological characterization of human BK channels in mammalian cells

For single channel recordings from Chinese hamster ovary (CHO) cells, *KCNMA1* cDNA (wild type and mutant) were subcloned into a pIRES2-EGFP vector (Clontech) with the restriction enzymes NheI and XhoI. CHO cells were plated onto coverslips in 12 well Falcon plates, and transfected (0.8 μ g DNA/well) using lipofectamine (4 μ l/well; Life Technologies) 6-12 hours before recording. Coverslips were placed in a recording chamber on an inverted light microscope (Axiovert 100, Zeiss) and superfused with Ringer solution at 2 ml/min. Transfected cells were selected by visualizing GFP fluorescence. Patch-clamp recordings were made in the inside-out configuration with borosilicate glass electrodes fabricated using a P-97 microelectrode puller (Sutter Instr.). Microelectrodes (20-100 M Ω) were filled with a solution containing (in mM): 144 KCl, 16 NaCl, 2 MgCl₂, 2 TES, 11 glucose, 0.065 CaCl₂, and 0.08 EGTA. After obtaining a patch, the electrode tip was moved into a separate minichamber (3), and the inside face of the patch was exposed to the same solution (flow rate 1 ml/min) in which the amount of CaCl₂ was varied to give a free calcium concentration of 1, 2, 5, 10, 20, 50, or 100 μ M (calculated using Webmaxc; www.stanford.edu/~cpatton/maxc.html). Recordings of single channel currents were made at room temperature in voltage clamp from a holding potential of -60 mV to test potentials from -100 to +100mV (steps of 20 mV for 3 seconds each). Currents were low pass filtered at 2 kHz, and digitized at a rate of 10 kHz using an Axopatch 1D amplifier, Digidata 1322a A/D converter and

PClamp software (Axon Instruments). The open channel probability was analyzed using Clampfit software (Axon Instruments) and the data were fit to the Boltzmann and Hill equations using Origin Software (OriginLab Corp).

Table 2**Genomic structure of human *KCNMA1* gene**

5

Acceptor sites (Intron/Exon)	Exon (bp)	Donor sites (Exon/Intron)
	1 (379)	AAGACGAAGG(SEQ ID NO: 5)/taacgcgcgc(SEQ ID NO: 6)
ctctgttgcc(SEQ ID NO: 7)/AGGAGGCCCA(SEQ ID NO: 8)	2 (166)	GAGTCCTGGT(SEQ ID NO: 9)/gagctccac(SEQ ID NO: 10)
ttcttttaca(SEQ ID NO: 11)/GGTTGTCTTA(SEQ ID NO: 12)	3 (63)	ATTCATCAAAA(SEQ ID NO: 13)/gtgagtattc(SEQ ID NO: 14)
tgtctaataag(SEQ ID NO: 15)/CCCAATAGAA(SEQ ID NO: 16)	4 (94)	CGGCTTGCGG(SEQ ID NO: 17)/gtaagttgt(SEQ ID NO: 18)
ctttcccca(SEQ ID NO: 19)/GTTTATTGCA(SEQ ID NO: 20)	5 (113)	TTGGCTTGGT(SEQ ID NO: 21)/aagtcagcct(SEQ ID NO: 22)
ctttttcca(SEQ ID NO: 23)/GGTTTGAGAT(SEQ ID NO: 24)	6 (81)	AACAAGGTAA(SEQ ID NO: 25)/gatgttctc(SEQ ID NO: 26)
ttctgtgcac(SEQ ID NO: 27)/AGTAATTCCA(SEQ ID NO: 28)	7 (81)	CCATTGTGTG(SEQ ID NO: 29)/agtaaccttg(SEQ ID NO: 30)
tccatttg(SEQ ID NO: 31)/TTTAGGTGGA(SEQ ID NO: 32)	8 (177)	GGGGGACTGG(SEQ ID NO: 33)/taaaaaaaaa(SEQ ID NO: 34)
tatcttctca(SEQ ID NO: 35)/GGCCATGTTT(SEQ ID NO: 36)	9 (94)	TGGAAGAAAG(SEQ ID NO: 37)/taagtatgcc(SEQ ID NO: 38)
tcctgttcag(SEQ ID NO: 39)/GCACATTGTG(SEQ ID NO: 40)	10 (111)	TTCTTCACAA(SEQ ID NO: 41)/gtaagagcct(SEQ ID NO: 42)
ccctgtgtag(SEQ ID NO: 43)/CATCTCCCCC(SEQ ID NO: 44)	11 (106)	AAGAGTCAAG(SEQ ID NO: 45)/gtaaggagga(SEQ ID NO: 46)
tcggtgtctt(SEQ ID NO: 47)/AGATAGAGTC(SEQ ID NO: 48)	12 (92)	GAGGTAACTC(SEQ ID NO: 49)/tggggcgct(SEQ ID NO: 50)
tttcttttc(SEQ ID NO: 51)/AGAGTAATCT(SEQ ID NO: 52)	13 (73)	CACAACAAGG(SEQ ID NO: 53)/taggagtgcg(SEQ ID NO: 54)
ttctttccc(SEQ ID NO: 55)/AGGCCCATCT(SEQ ID NO: 56)	14 (158)	ATTCATAAAG(SEQ ID NO: 57)/gtaacgtctc(SEQ ID NO: 58)
cctttcctt(SEQ ID NO: 59)/AGATTGAGGA(SEQ ID NO: 60)	15 (113)	TGTTTGTGAG(SEQ ID NO: 61)/taagtgacc(SEQ ID NO: 62)t
ctcattgcag(SEQ ID NO: 63)/GCTGTGTTTT(SEQ ID NO: 64)	16 (69)	GAGAGAGCCG(SEQ ID NO: 65)/gtaagtttgc(SEQ ID NO: 66)
ttgttctact(SEQ ID NO: 67)/GAGCAGTATA(SEQ ID NO: 68)	17 (94)	AGTTAAAAGG(SEQ ID NO: 69)/taagagtta(SEQ ID NO: 70)
ctcttttccc(SEQ ID NO: 71)/AGGGCATTTT(SEQ ID NO: 72)	18 (79)	TGCAAACGGC(SEQ ID NO: 73)/gtaagtagtg(SEQ ID NO: 74)
gtatttcag(SEQ ID NO: 75)/TTGAAGATGA(SEQ ID NO: 76)	19 (95)	GCTGATGAGG(SEQ ID NO: 77)/taaggagagg(SEQ ID NO: 78)
ttttctccc(SEQ ID NO: 79)/AGGCATGACC(SEQ ID NO: 80)	20 (126)	AGTCATCCTG(SEQ ID NO: 81)/gtgagtggagg(SEQ ID NO: 82)
ccctgttgta(SEQ ID NO: 83)/GACTCGAAGT(SEQ ID NO: 84)	21 (227)	ATATTGCCTG(SEQ ID NO: 85)/taagtccaag(SEQ ID NO: 86)
tatgccaag(SEQ ID NO: 87)/GGTACGCCAT(SEQ ID NO: 88)	22 (194)	ATTCCCAAGG(SEQ ID NO: 89)/taaggacagc(SEQ ID NO: 90)

ctctgtttcc(SEQ ID NO: 91)/TTCCCACTAG(SEQ ID NO: 92)	23 (124)	ACTGAACTAG(SEQ ID NO: 93)/gtaggggtca(SEQ ID NO: 94)
tatgtttttt(SEQ ID NO: 95)/CTAGTGAACG(SEQ ID NO: 96)	24 (135)	CATGAGCGCG(SEQ ID NO: 97)/gtgagttatc(SEQ ID NO: 98)
tccttcccca(SEQ ID NO: 99)/GACGTACTTC(SEQ ID NO: 100)	25 (197)	GACTTAGGGG(SEQ ID NO: 101)/tgagtccaca(SEQ ID NO: 102)
ctgtctccca(SEQ ID NO: 103)/GGATGGTGGT(SEQ ID NO: 104)	26 (122)	ACAAAGAGGT(SEQ ID NO: 105)/gagtattggt(SEQ ID NO: 106)
ttccctgtcc(SEQ ID NO: 107)/AGGTATGTCA(SEQ ID NO: 108)	27 (258)	TTGATATGTG(SEQ ID NO: 109)/tatccaccgc(SEQ ID NO: 110)

Table 3**PCR primers for amplification of *KCNMA1* exons and mutational analysis**

Exon(s)	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	AT
1	AGTAGCAGCAATATGGCTGTTG(SEQ ID NO: 111)	AGAAGCGGTGGGGCTGGCGCAG (SEQ ID NO: 112)	60°C
2	GGTTCCTTATGGGTAGAGCATG(SEQ ID NO: 113)	CTCATAAGCAAAGCCACCTTGG (SEQ ID NO: 114)	58°C
3	AAACATCCTGAGGTCCAACCTC(SEQ ID NO: 115)	ATCAATGTAAAGGCTCATGATTG (SEQ ID NO: 116)	58°C
4	GATGATACTACCTGTCATTTACC(SEQ ID NO: 117)	GACTGCGAGAGCAGAGGCTG (SEQ ID NO: 118)	58°C
5	TGCCAGCACCATGCTTCTCATG(SEQ ID NO: 119)	ATTCTGTCCTTCAGCCATGACTC (SEQ ID NO: 120)	58°C
6	AAATCAAATGGCAGACTGTGAAC(SEQ ID NO: 121)	CAAGCACACCTGATATTTGCAAC (SEQ ID NO: 122)	58°C
7	TGCTGTTACCCTGTAAGGCAG(SEQ ID NO: 123)	AGAGGGTCTTGTTCAGGCAAG (SEQ ID NO: 124)	58°C
8	GCATGTATACACTTCAATAGCATG(SEQ ID NO: 125)	GCTTCTAGTGCAGAATACAGTC (SEQ ID NO: 126)	58°C
9	TATACCTTTGCAGGCCTAATCC(SEQ ID NO: 127)	GAGAGGATTCTACCGCAGCAG(SEQ ID NO: 128)	58°C
10	TGCAGGGTCCTGTCCGTGGT(SEQ ID NO: 129)	ACCTCCACTCTTACAGACATTC (SEQ ID NO: 130)	58°C
11	CTGTGGAAATTAGAGAGGAAGG(SEQ ID NO: 131)	TGGCCAACCTAGGAGGTGTG(SEQ ID NO: 132)	58°C
12	GAGACTACACTCGAGCATGGC(SEQ ID NO: 133)	GATAGTCCTCTGCAGAAGATCC (SEQ ID NO: 134)	58°C
13	CTGCCTCCTACCTCAGTCCC(SEQ ID NO: 135)	AGGAAGAAGAGGCTGGACCTG (SEQ ID NO: 136)	58°C
14	CCCAGAAGAAATGTAAACAATTG (SEQ ID NO: 137)	TAAGCTGTGCTCTCTACTCAAC (SEQ ID NO: 138)	53°C
15	AAGAGTGTAACCTCAGGTTTCCC(SEQ ID NO: 139)	TCCAACTGTACCCAGCAGAGG(SEQ ID NO: 140)	58°C
16	CTCTGGTCATTCAGAAGAACTG(SEQ ID NO: 141)	TCAGAACGCACTCTCACCATC(SEQ ID NO: 142)	58°C
17	ACAACAGTATGGTGTAGCCTTC(SEQ ID NO: 143)	CCGTGGGTCAAGGTGTCTAC(SEQ ID NO: 144)	58°C
18	CGTGGAGGAAATGTGGTACTC(SEQ ID NO: 145)	CATACTCAAGAAACTCTCGATG (SEQ ID NO: 146)	53°C
19	GCCTTGAGTGTGTGCTTTG(SEQ ID NO: 147)	CTTTAAGAAGCCATCTAACGATC (SEQ ID NO: 148)	58°C
20	CACATCCTCTGAGATGTAATTC(SEQ ID NO: 149)	CAGTATTATCCCCTGTCCAAC (SEQ ID NO: 150)	58°C

21	TAGCACATAGTAAGCTCTCAGC(SEQ ID NO: 151)	AAGCTACAACCTATTATATCCATCC (SEQ ID NO: 152)	58°C
22	TCCCTCTCCTCTCACTTTTGC(SEQ ID NO: 153)	TACTGAGTGAAGGATATCCCTC (SEQ ID NO: 154)	58°C
23	GTTGTATGTATATAGTGTACTCTG(SEQ ID NO: 155)	AAACCAACCTCCTCATATCCTG (SEQ ID NO: 156)	58°C
24	CCTGGTCTCCCTCTGGCTTC(SEQ ID NO: 157)	TGCTCTATTGGCACATCCTCC(SEQ ID NO: 158)	58°C
25	TTGGATGTCGGCTGTCATGAC(SEQ ID NO: 159)	ATCTGATACCACGTTCTTCAGG (SEQ ID NO: 160)	59°C
26	CCCTGCTGTCCCTTGATTTTC(SEQ ID NO: 161)	CCTTTACAGTAGGGGACAGG(SEQ ID NO: 162)	59°C
27	GCTTTTGGTTCAGAGAGAGTTG(SEQ ID NO: 163)	GTTGGAAACACCAACTGGGG(SEQ ID NO: 164)	58°C

Having described the invention the following is claimed:

1. A method of identifying a patient that has, or is at risk of developing general epilepsy and/or paroxysmal dyskinesia, the method comprising:
detecting an alteration of the *KCNMA1* gene, the alteration disrupting the function of the calcium-activated BK potassium channel alpha subunit so as to produce general epilepsy and/or paroxysmal dyskinesia.
2. The method of claim 1, the alteration comprising a mutation in the coding region of the *KCMNA1* gene, the mutation increasing Ca^{2+} sensitivity of the calcium-activated BK channel.
3. The method of claim 1, the mutation resulting in at least one of a insertion, deletion, point mutation, or inversion of nucleic acids in at least one exon of the *KCMNA1* gene.
4. The method of claim 1, the mutation resulting in at least one of an insertion, deletion, point mutation, or inversion of nucleic acids in exon 10 of the *KCMNA1* gene.
5. The method of claim 4, the mutated KCMNA1 gene having a nucleotide sequence corresponding to SEQ ID NO: 2.
6. The method of claim 4, the mutation of the *KCMNA1* gene resulting in the substitution of aspartic acid for glycine of amino acid 434 of a wild type KCMNA1 corresponding to SEQ ID NO: 3.

7. The method of claim 1, detection of the alteration being performed by amplifying at least one of exons 1-27 of the *KCMNA1* gene by polymerase chain reaction and analyzing the amplification products produced by the polymerase chain reaction for mutations.

8. The method of claim 8 the analyzing of the amplification products being performed by heteroduplex or single strand conformation polymorphism analysis.

9. A method of identifying a patient that has, or is at risk of developing general epilepsy and/or paroxysmal dyskinesia, the method comprising:

providing a nucleic acid sample from the individual, the nucleic acid comprising nucleic acid sequence corresponding to a *KCNMA1* gene;

determining if the nucleic acid sequence corresponding to at least one of a *KCNMA1* gene of the patient are mutated such that the mutation results in a gain of function of the calcium-activated BK potassium channel alpha subunit encoded by the *KCNMA1* gene.

10. The method of claim 9, the alteration comprising a mutation in the coding region of the *KCMNA1* gene, the mutation increasing Ca^{2+} sensitivity of the calcium-activated BK channel.

11. The method of claim 9, the mutation resulting in at least one of a insertion, deletion, point mutation, or inversion of nucleic acids in at least one exon of the *KCMNA1* gene.

12. The method of claim 9, the mutation resulting in at least one of insertion, deletion, point mutation, or inversion of nucleic acids in exon 10 of the *KCMNA1* gene.

13. The method of claim 9, the mutated *KCMNA1* gene having a nucleotide sequence corresponding to SEQ ID NO: 2.

14. The method of claim 9, the mutation of the *KCMNA1* gene resulting in the substitution of aspartic acid for glycine of amino acid 434 of a wild type *KCMNA1* gene corresponding to SEQ ID NO: 3.

15. The method of claim 14, detection of the alteration being performed by amplifying at least one of exons 1-27 of the *KCMNA1* gene by polymerase chain reaction and analyzing the amplification products produced by the polymerase chain reaction for mutations.

16. The method of claim 15, the analyzing of the amplification products being performed by heteroduplex or single strand conformation polymorphism analysis.

17. An isolated nucleic acid encoding a mutant or variant calcium-activated BK potassium channel alpha subunit, the isolated nucleic acid comprising a nucleic acid sequence corresponding to a variant of SEQ ID NO: 1, the variant of SEQ ID NO: 1, comprising an alteration of the nucleic acid sequence which disrupts the function of the calcium-activated BK potassium channel alpha subunit so as to produce general epilepsy and/or paroxysmal dyskinesia.

18. The isolated nucleic acid of claim 17, the alteration comprising an insertion, deletion, and/or substitution of a nucleic acid of SEQ ID NO: 1.

19. The isolated nucleic acid of claim 17, having a nucleotide sequence corresponding to SEQ ID NO: 2.

20. An isolated polypeptide, the isolated polypeptide being a mutant or variant calcium-activated BK potassium channel alpha subunit, wherein the mutation has occurred such that the polypeptide has an amino acid sequence corresponding to SEQ ID NO: 4.

21. A method of identifying agents that can be used to treat individuals having or at risk of developing general epilepsy and/or paroxysmal dyskinesia;
providing a transgenic animal with an alteration of the *KCNMA1* gene, the alteration disrupting the function of the calcium-activated BK potassium channel alpha subunit so as to produce general epilepsy and/or paroxysmal dyskinesia in the animal,
administering at least one agent to the animal to modulate the function of the BK channel.

22. The method of claim 19, the agents comprising a BK channel blocking agents.

23. The method of claim 22, the alteration of the *KCMNA1* gene comprising a mutation in the coding region of the *KCMNA1* gene, the mutation increasing Ca^{2+} sensitivity of the calcium-activated BK channel.

24. The method of claim 23, the mutation resulting in at least one of a insertion, deletion, point mutation, or inversion of nucleic acids in at least one exon of the *KCMNA1* gene.

25. The method of claim 23, the mutation resulting in at least one of a insertion, deletion, point mutation, or inversion of nucleic acids in exon 10 of the *KCMNA1* gene.

26. The method of claim 23, the mutated KCMNA1 gene having a nucleotide sequence corresponding to SEQ ID NO: 2.

27. The method of claim 23, the mutation of the *KCMNA1* gene resulting in the substitution of aspartic acid for glycine of amino acid 434 of a wild type KCMNA1 gene corresponding to SEQ ID NO: 3.

28. A method of treating general epilepsy and/or paroxysmal dyskinesia associated with a mutation of a calcium-activated BK potassium channel alpha subunit, the method comprising:

administering an agent comprising selective agonist, antagonist, and/or modulator of the calcium-activated BK potassium channel alpha subunit when the calcium-activated BK potassium channel alpha subunit has undergone a mutation event that results in general epilepsy and/or paroxysmal dyskinesia.

29. The method of claim 28, the agent comprising a BK channel blocking agent.

30. The method of claim 29, the agent comprising at least one of paxilline, charybdotoxin, iberiotoxin, penitrem A, tetraethyl ammonium chloride (TEA), apamin, clotrimazole, dequalium chloride, iberiotoxin, neuropeptide Y, or tityustoxi.

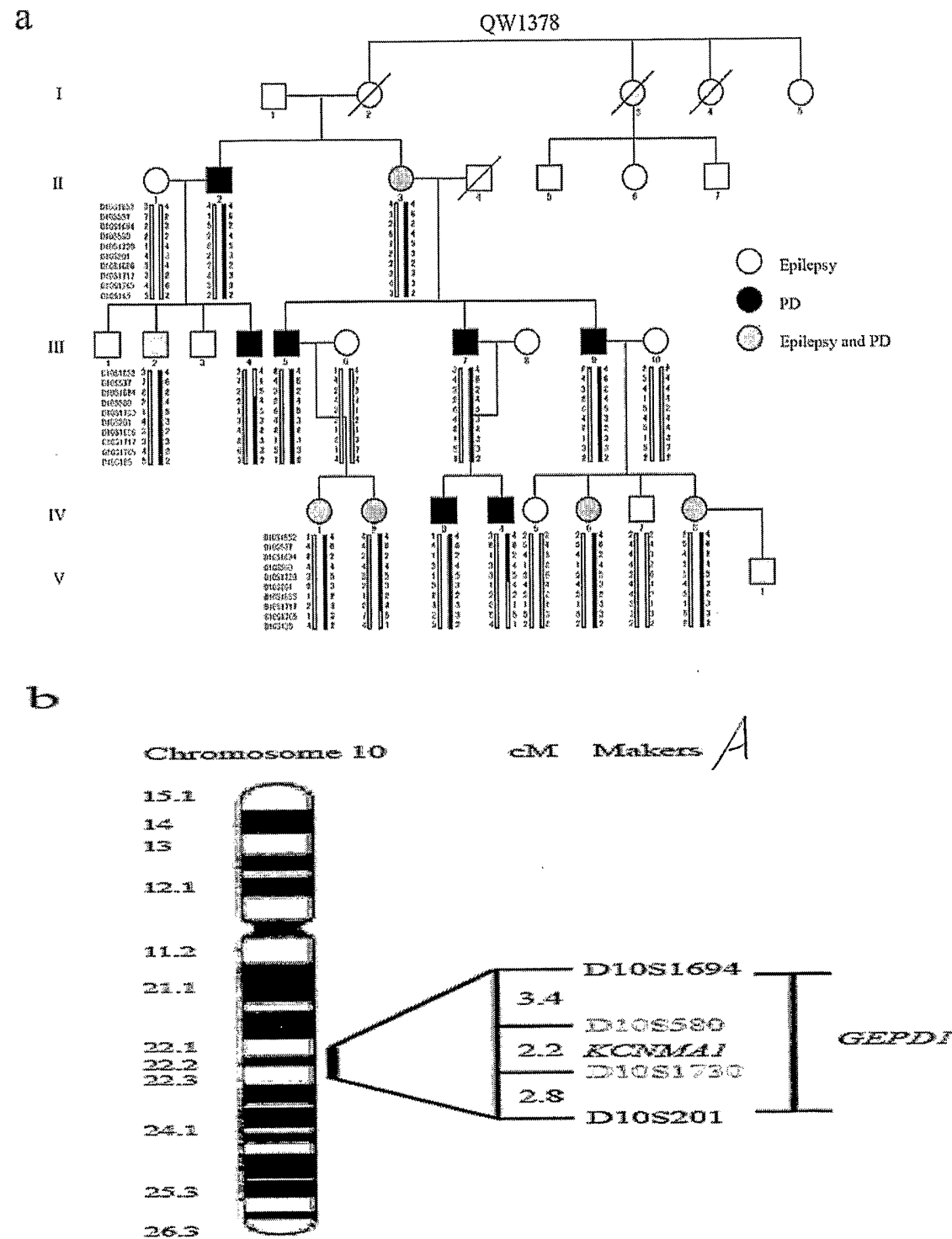


Fig. 1

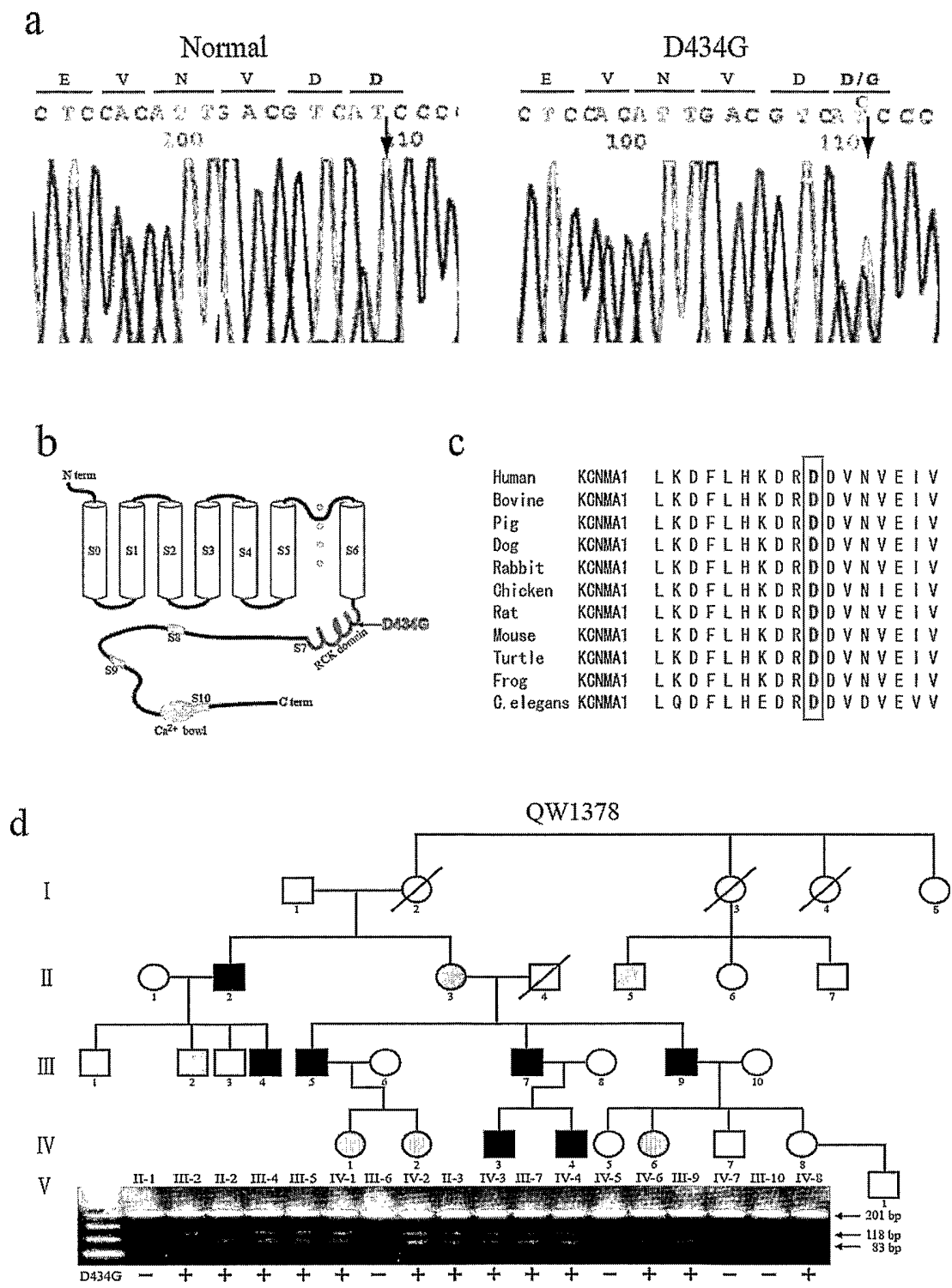


Fig. 2

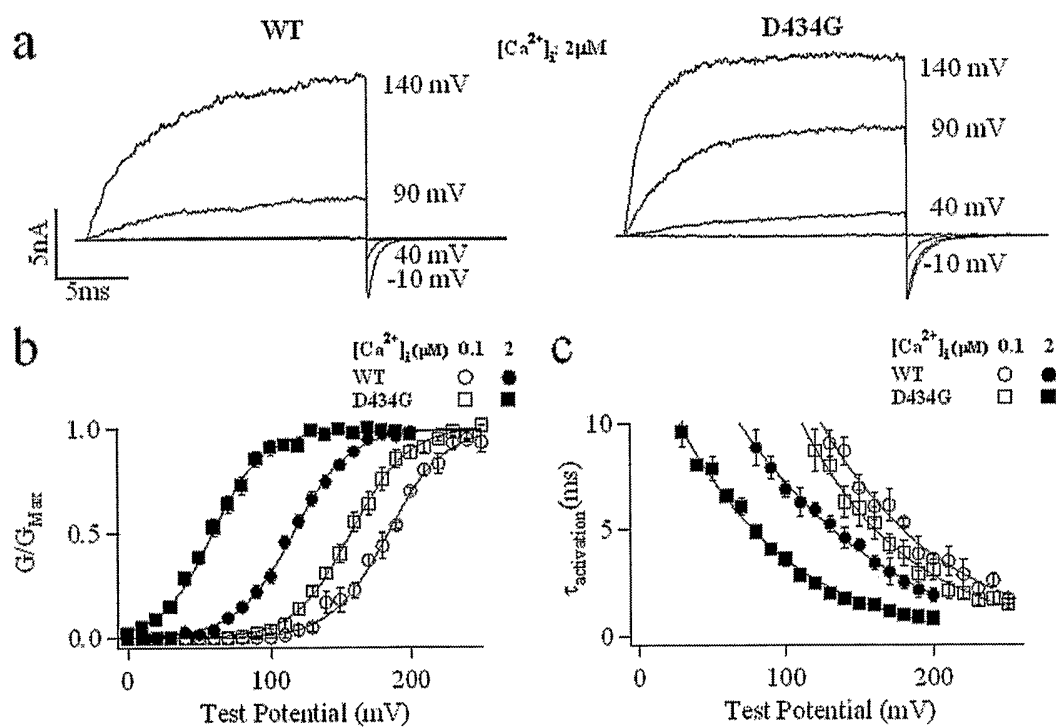


Fig. 3

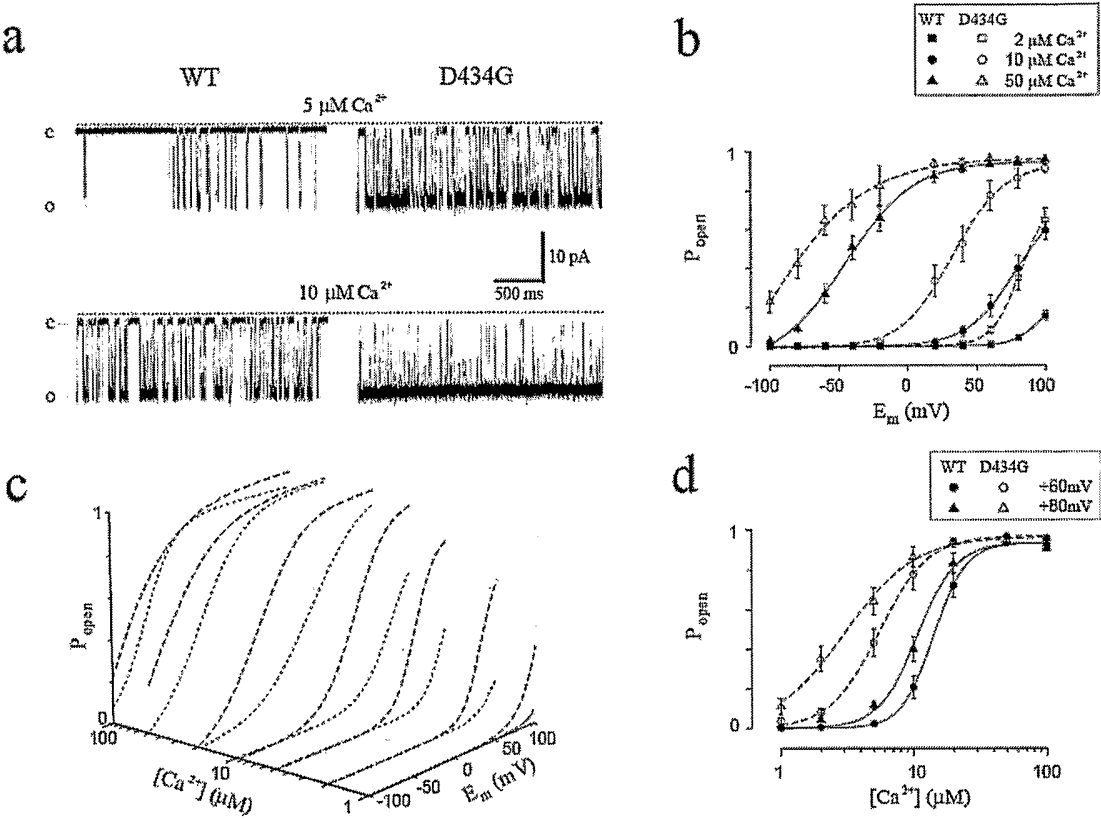


Fig. 4

5/5

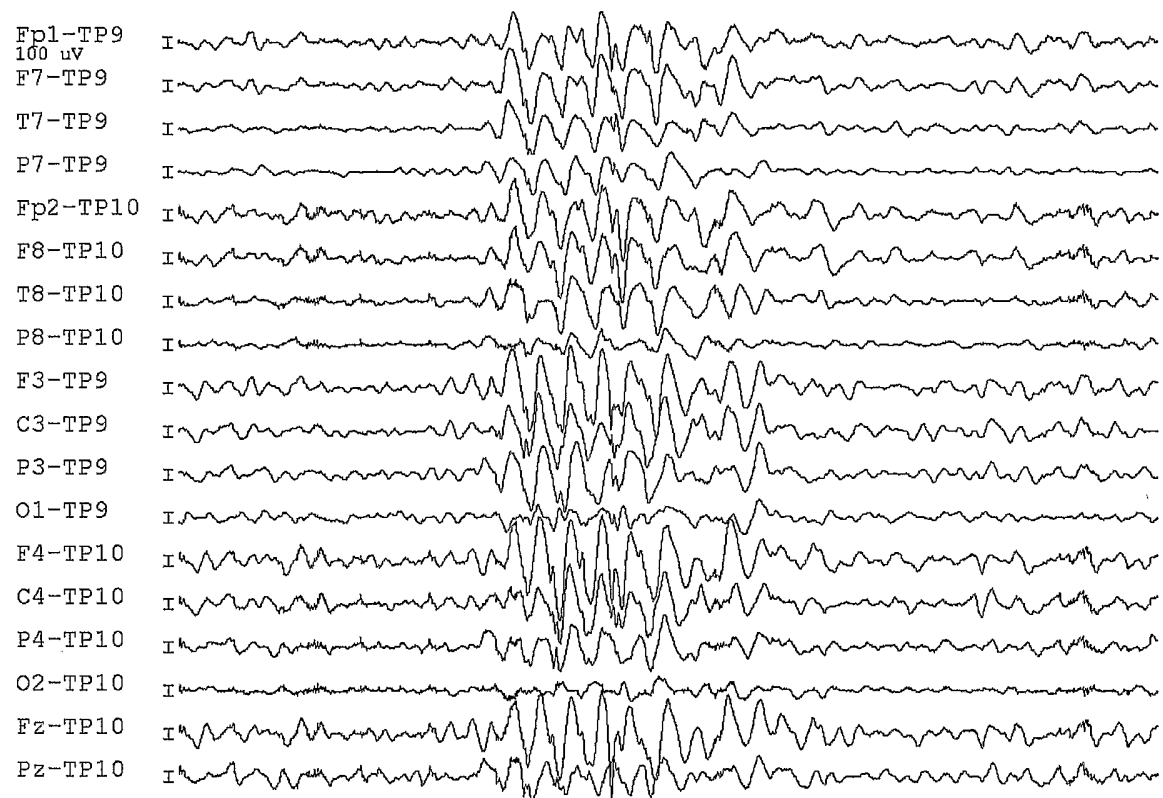


Fig. 5

SEQUENCE LISTING

<110> Wang, Qing

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AND EPILEPSY

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Arg Asp Asp Val Asn Val Glu Ile Val Phe Leu His Asn Ile Ser Pro
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Lys Leu Lys Leu Leu Met Ile Ala Ile Glu Tyr Lys Ser Ala Asn Arg		
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Lys His Ile Val Phe Val Gly Ser Ile Glu Tyr Leu Lys Arg Glu Trp		
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Glu Thr Leu His Asn Phe Pro Lys Val Ser Ile Leu Pro Gly Thr Pro		
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Cys Val Ile Leu Ser Ala Asn Gln Asn Asn Ile Asp Asp Thr Ser Leu		
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Gln Asp Lys Glu Cys Ile Leu Ala Ser Leu Asn Ile Lys Ser Met Gln		
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Lys Met Asp Ala Leu Ile Ile Pro Val Thr Met Glu Val Pro Cys Asp
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Tyr Leu Trp Thr Val Cys Cys His Cys Gly Gly Lys Thr Lys Glu Ala
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770	775	780

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Asn	Leu	Val	Met	Pro	Leu	Arg	Ala	Ser	Asn	Phe	His	Tyr	His	Glu	Leu	805	810	815	
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Cys	Val	Ile	Leu	Ser	Ala	Asn	Gln	Asn	Asn	Ile	Asp	Asp	Thr	Ser	Leu	865	870	875	880
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Lys Ser Ser Ser Val His Ser Ile Pro Ser Thr Ala Asn Arg Gln		
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