Mutations in Kir2.1 Cause the Developmental and Episodic Electrical Phenotypes of Andersen's Syndrome

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Summary

Andersen's syndrome is characterized by periodic paralysis, cardiac arrhythmias, and dysmorphic features. We have mapped an Andersen's locus to chromosome 17q23 (maximum LOD = 3.23 at θ = 0) near the inward rectifying potassium channel gene *KCNJ2*. A missense mutation in *KCNJ2* (encoding D71V) was identified in the linked family. Eight additional mutations were identified in unrelated patients. Expression of two of these mutations in *Xenopus* oocytes revealed loss of function and a dominant negative effect in Kir2.1 current as assayed by voltage-clamp. We conclude that mutations in Kir2.1 cause Andersen's syndrome. These findings suggest that Kir2.1 plays an important role in developmental signaling in addition to its previously recognized function in controlling cell excitability in skeletal muscle and heart.

Introduction

Andersen's syndrome (AS) is a rare disorder characterized by periodic paralysis, cardiac arrhythmias, and dysmorphic features (Figure 1) (Canun et al., 1999; Sansone et al., 1997; Tawil et al., 1994). The dysmorphology includes short stature, scoliosis (curvature of the spine), clinodactyly (permanent lateral or medial curve of a finger or toe), hypertelorism (wide-set eyes), small or prominent ears that are low set or slanted, micrognathia (small chin), and broad forehead. AS occurs either sporadically or as an autosomal dominant trait. In AS families, expression of the characteristic traits is highly variable (Figure 2A). Thus, it is likely that the AS protein plays a complex role in development and cell excitability, having some redundancy with other proteins. This disorder is the first to demonstrate a predicted link between muscle and cardiac electrical phenotypes and also to provide an exciting link between electrical episodic and developmental phenotypes.

The periodic paralyses and nondystrophic myotonias are a group of muscle disorders characterized by abnormal muscle relaxation (myotonia). This myotonia results from muscle hyperexcitability that sometimes transitions to inexcitability resulting in episodic weakness. Ventricular tachyarrhythmias are analogous to myotonia of skeletal muscle in that hyperexcitability leads to an abnormal series of heart contractions that can lead to death. Approximately 300,000 Americans die of cardiac arrhythmias each year (Kannel et al., 1987; Willich et al., 1987). The electrophysiological features of such diseases suggest an underlying defect in membrane excitability. The first ion channel mutations contributing to an episodic disorder were characterized less than a decade ago when mutations in *SCN4A*, which encodes a voltage-gated sodium channel, were shown to cause hyperkalemic periodic paralysis (Ptacek et al., 1991; Rojas et al., 1991). This muscle disease formed the basis of the growing group now known as the channelopathies and led to predictions that cardiac dysrhythmias, epilepsies,
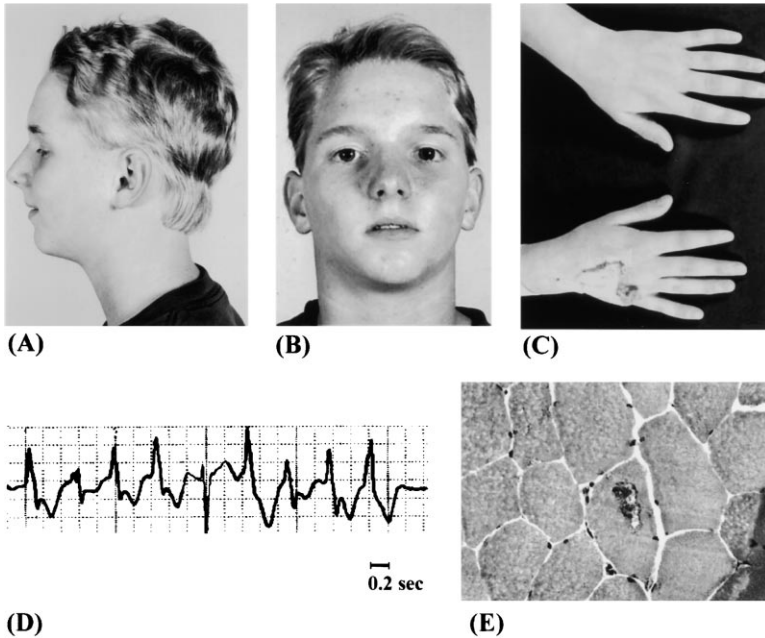
Figure 1. Andersen's Syndrome Is Characterized by Dysmorphic Features, Cardiac Arrhythmias, and Periodic Paralysis

(A and B) Andersen's patient exhibiting low set ears, hypertelorism, micrognathia, and (C) clinodactyly of the fifth digits. (D) ECG rhythm strip from an Andersen's patient demonstrating short runs of polymorphic ventricular tachycardia. (E) Muscle biopsy of an Andersen's patient exhibiting tubular aggregates commonly seen in periodic paralysis patients.

and some migraine conditions would be caused by mutations in homologous genes (Ptacek et al., 1991). Similarities between these different episodic phenotypes suggested similar molecular bases of these disorders. The occurrence of both periodic paralysis and long QT (LQT) in Andersen's syndrome strongly supported this

hypothesis (Tawil et al., 1994). Periodic paralysis has been associated with mutations in voltage-gated K^+, Na^+, Ca^{2+}, and Cl^- channels (Jen and Ptacek, 2001), while LQT syndrome (LQTS) has been associated with mutations in voltage-gated K^+ and Na^+ channels (Keating and Sanguinetti, 2001). To date, no human disorders involving cardiac and skeletal muscle have been attributed to mutations in inward rectifying K^+ (Kir) channels.

In this report, we present extensive genetic and functional data demonstrating that *KCNJ2*, encoding the inward rectifying K^+ channel Kir2.1, is a major Andersen's syndrome gene. We propose that dominant negative mutations of Kir2.1 channel function are responsible for the dysmorphic features, periodic paralysis, and cardiac arrhythmias of AS. Importantly, mutations in Kir2.1 could account for some unexplained cases of periodic paralysis and cardiac arrhythmias. Not only does this study help support the predictions that mutations in Kir channels cause episodic phenotypes, but it also supports the role of this ion channel in providing developmental signals.

(A)

(B)

A→T

(C)

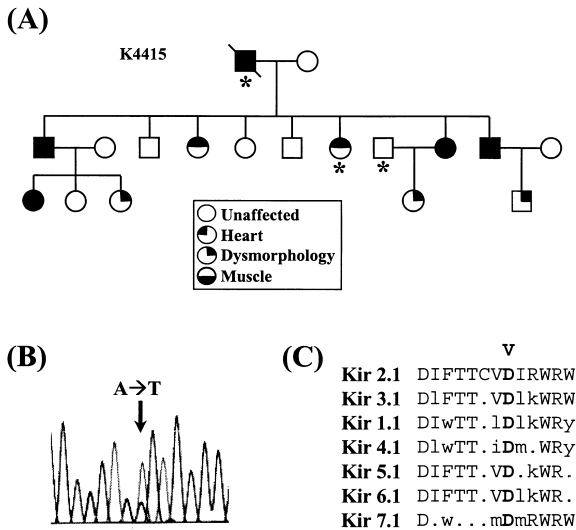|	
	v
Kir 2.1	DIFTTCV**D**IRWRW
Kir 3.1	DlFTT.V**D**lkWRW
Kir 1.1	DIwTT.l**D**lkWRy
Kir 4.1	DlwTT.i**D**m.WRy
Kir 5.1	DIFTT.V**D**.kWR.
Kir 6.1	DIFTT.V**D**lkWR.
Kir 7.1	D.w...m**D**mRWRW

Figure 2. Pedigree of Kindred 4415 Exhibiting Variable Expressivity and Associated *KCNJ2* Mutation

(A) Females are denoted with circles and males with squares. An "*" denotes an individual that was not included in the genome-wide linkage screen.

(B) Sequence chromatogram of an affected individual with an A to T transversion corresponding to the D71V mutation.

(C) Amino acid alignment of one subunit from each of the seven members of the inward rectifying K^+ channels. The mutation is denoted above the alignment. Lowercase letters denote conservative amino acid changes, whereas a "." denotes a nonconservative amino acid change.

Results

Patients with Andersen's Have Variable Expressivity
A total of 16 unrelated Andersen's syndrome kindreds were identified who met the defined diagnostic criteria (see Experimental Procedures). Most kindreds were small, consisting of one to three affected individuals. Affected individuals showed marked variability in the phenotypic expression of the disease. While some individuals, typically including the index cases, manifested the full Andersen's triad (Figure 1), other affected individuals demonstrated only one or two of the major characteristics of this disorder. Dysmorphic features ranged from negligible deformities of the digits to very prominent facial dysmorphisms (Figure 1). Cardiac manifestations included asymptomatic LQT, ventricular ectopy, bi-directional ventricular tachycardia, syncope, recurrent *torsades de*

pointes, and cardiac arrest requiring treatment with an implantable defibrillator. Attacks of paralysis were associated with hypo-, hyper-, or normokalemia. Although serum potassium levels during attacks differed among kindreds, they were consistent within an individual kindred.

An Andersen's Syndrome Allele Is Located on Chromosome 17q23

Approximately 400 polymorphic markers were analyzed across the entire genome in 15 individuals of kindred 4415 to identify the Andersen's locus (Figure 2A). Individuals were classified as affected in this analysis if they exhibited one of the three main characteristics of Andersen's syndrome (periodic paralysis, cardiac arrhythmias, and dysmorphic features). One marker (D17S949) from the set of 400 maximized at $\theta = 0$ with a LOD score of 3.23. The simulated maximum LOD score for this kindred for a 5-allele system was 3.21 at $\theta = 0$. Marker D17S787 set the proximal recombinant boundary, whereas D17S784 set the distal recombinant boundary. This region corresponds to a genetic region of over 40 cM on chromosome 17q23.

KCNJ2, *CACNG1*, and *SCN4A* Are Candidate Genes for Andersen's Syndrome

To identify putative candidate genes located within our linked region, we examined the mapviewer database from the National Center for Biotechnology Information website. Previous findings that periodic paralysis and LQTS were associated with mutations in ion channels led us to predict that the AS gene also encoded an ion channel. Chromosome 17q23 contigs contained three ion channel genes within our linked region, Kir channel *KCNJ2*, calcium channel *CACNG1*, and sodium channel *SCN4A*. Because *SCN4A* had already been shown to be responsible for periodic paralysis without heart or developmental problems (Ptacek et al., 1991), and the *CACNG1* gene product is not detected in heart, *KCNJ2* was examined first. Due to the known function and expression pattern of Kir2.1, *KCNJ2* was considered an excellent candidate gene for Andersen's syndrome.

D71V Segregates with Andersen's Syndrome

The coding region of *KCNJ2*, contained within one exon, was PCR amplified and sequenced in all individuals from kindred 4415 from whom DNA was available. This excludes the deceased individual in the first generation. An A to T transversion corresponding to the D71V mutation was identified in all affected individuals but not in any unaffected family members (Figures 2A, 2B, and 3). This mutation was not found in 100 unaffected and unrelated individuals.

Eight Additional Mutations Have Been Identified in Andersen's Syndrome Probands

We subsequently examined 15 additional unrelated AS probands for mutations in *KCNJ2* (Figure 4). As with kindred 4415, the coding region of *KCNJ2* was PCR amplified and sequenced. In total, 9 mutations were identified in 13 probands (Figures 4 and 5). Three of the 16 families examined did not have any mutations in the coding region of *KCNJ2*. No discernible difference was observed in the clinical manifestations between kindreds with
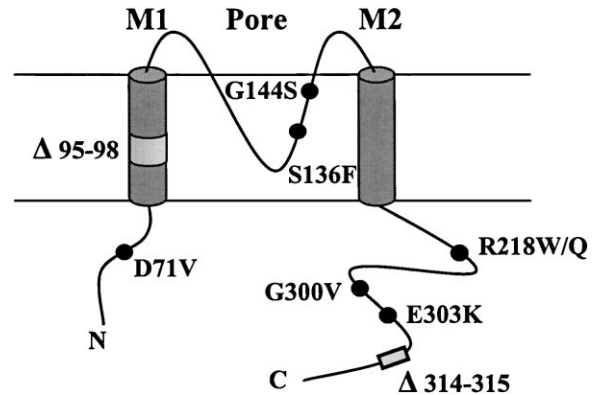

Figure 3. Structure of Kir2.1 and Inward Rectifying K+ Channels
The locations of identified Andersen's syndrome mutations are represented on the structure.

Kir2.1 mutations and those without a definable mutation. The mutations occur at highly conserved residues (Figure 5). Seven of the nine mutations are missense mutations consisting of the following changes (numbering is based on *KCNJ2* sequence with GenBank accession number AF153819): D71V (A440T), S136F (T635C), G144S (G658A), R218W (C880T), R218Q (G881A), G300V (G1127T), and E303K (G1135A). Two mutations are in-frame deletions: Δ95–98 (bp 513–524) and Δ314–15 (bp 1167–1172). All substitution mutations were checked in over 100 unaffected unrelated individuals by SSCP or mutation-specific PCR analysis (Table 1) and were not found in this panel. R218W occurred in four families, and G300V is present in two families. All other mutations were only identified in single families. At least three of the changes represent de novo mutations (G144S, R218W (three events), and Δ314–15). Only one polymorphism was identified in AS probands. This polymorphism is a silent mutation of C1374T in the codon for residue L382.

The mutations are located throughout the protein (Figure 3). D71V resides in the N terminus in the last position of a predicted α helix. This residue is just distal to the putative N-terminal interaction domain (Tucker and Ashcroft, 1999). Deletion Δ95–98 removes four residues of the M1 transmembrane segment. This might completely prohibit the M1 segment from being inserted into the membrane. S136F and G144S are both pore mutations. G144S is located in the first position of the highly conserved K+ channel signature sequence GYG. Several mutations reside in the C terminus. R218W and R218Q are located within the C-terminal interaction domain (Tinker et al., 1996), and G300V, E303K, and Δ314-15 are located in a region of unknown function.

D71V and R218W Mutations Cause Loss of Function and Result in a Dominant Negative Effect in Kir2.1

The ability of mutant Kir2.1 subunits to form functional homomultimeric channels was assessed by comparing oocytes injected with wild-type (WT) or mutant Kir2.1 cRNA (23 ng/oocyte). Injection of *WT Kir2.1* induced nearly instantaneous K+ currents that demonstrated
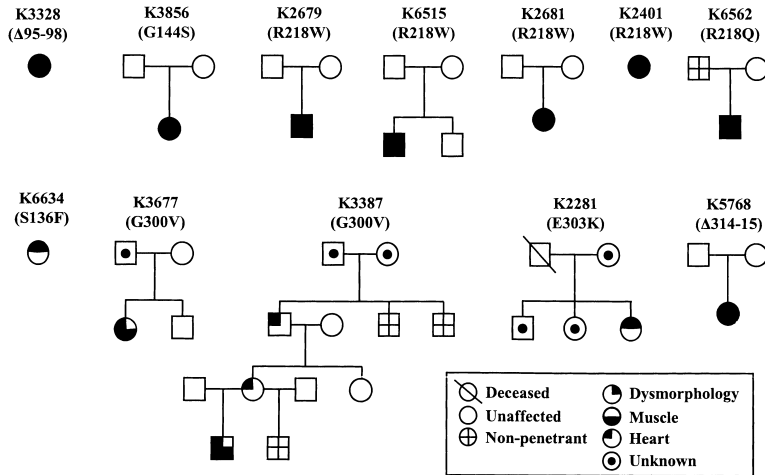
Figure 4. Additional AS Kindreds with Identified Mutations in Kir2.1

Females are denoted with circles and males with squares. The kindred number and mutation are denoted above each pedigree. De novo mutations occurred in kindreds 3856, 2679, 6515, 2681, and 5768. "Unknown" individuals are those for whom we have no clinical data.

strong inward rectification (see Supplemental Figures S1 and S2 at http://www.cell.com/cgi/content/full/105/4/511/DC1), as previously described (Raab-Graham et al., 1994). Inward rectification refers to the property that permits inward flux of K^+ ions at potentials negative to the K^+ equilibrium potential (E_K) more readily than outward flux at potentials positive to E_K. D71V and R218W mutant subunits failed to form functional homomultimeric channels. Injection of *D71V* or *R218W* cRNA did not induce detectable K^+ currents (Figure 6). Small endogenous currents, identical to those in H_2O-injected control oocytes, were recorded in oocytes injected with mutant cRNA (see Supplemental Figures S1 and S2 on *Cell* website).

AS is an autosomal-dominant disorder and, as such, affected individuals possess one normal and one mutant

KCNJ2 allele. To assess the ability of mutant Kir2.1 subunits to form functional heteromultimeric channels with WT subunits, we coinjected mutant (11.5 ng/oocyte) and WT Kir2.1 cRNA (11.5 ng/oocyte) and compared currents to those induced by injection of WT Kir2.1 cRNA (23 ng/oocyte). Coexpression of WT and D71V Kir2.1 subunits induced an inwardly rectifying K^+ current whose current amplitude was markedly reduced (Figure 6A). Current amplitude at -150 mV was -4.61 ± 0.37 μA for WT Kir2.1, compared to -0.26 ± 0.02 μA for coinjected WT and D71V. Assuming equal protein expression and random association of WT and mutant Kir2.1 subunits, 1/16 of the channels will be comprised of four WT subunits, whereas 15/16 of channels will contain one or more mutant subunits. The reduction in current induced by coexpression of WT and D71V subunits was approxi-
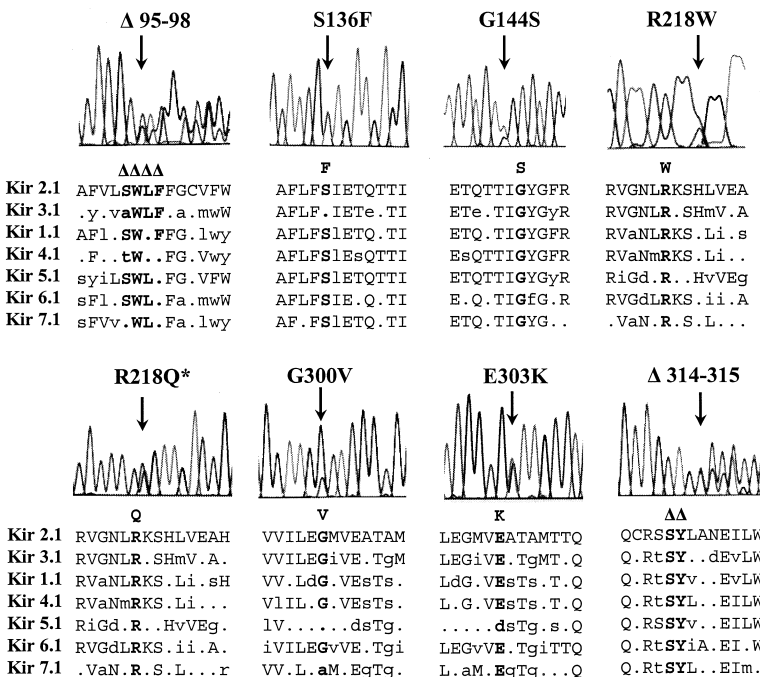

Figure 5. Mutations of Kir2.1 in Andersen's Patients Occur in Highly Conserved Residues

Chromatographs of the nucleotide sequence corresponding to each mutation are shown. *Nucleotide sequence shown is the reverse complement of coding sequence. Below each chromatograph is an alignment of the first member of all human Kir families. Mutant residues are denoted above each alignment. "Δ" marks a deletion of the residue below it. Lowercase letters denote conservative amino acid changes, whereas a "." denotes a nonconservative amino acid change.

Table 1. Primer Sequences and Mutational Analysis Conditions

Mutation		Primer Sequence	Method	T_a
D71V	F	5′ GGCAACGGTACCTCGCAGA 3′	MSP	62°C
	M	5′ GGGAACGATACCTCGCAGT 3′		
	R	5′ CAACCAAAACACACAGCCAAA 3′		
S136F	F	5′ CGGCTGCCTTCCTCTTCTC 3′	MSP	64°C
	M	5′ CGGGTGCCTTCCTCCTCTT 3′		
	R	5′ GTTTCTCTTCTTTGGCTTTGC 3′		
G144S*	F	5′ GCTTCACGGCTGCCTTCC 3′	SSCP	55°C
	R	5′ GTTTCTCTTCTTTGGCTTTGC 3′		
R218Q	F	5′ GGCGAGTGGGCAATCTTCG 3′	MSP	62°C
	M	5′ GGCGAGTAGGCAGTCTTCA 3′		
	R	5′ CTCAAATCATATAAAGGACTGTC 3′		
R218W	F	5′ GGCGAGTGGGCAATCTTCG 3′	MSP	57°C
	M	5′ GTCGCGAGTAGGCAATGTTT 3′		
	R	5′ CTCAAATCATATAAAGGACTGTC 3′		
G300V*	F	5′ AGGACATTGACAACGCAGAC 3′	SSCP	55°C
	R	5′ CATGGCAGTGGCTTCCACC 3′		
E303K	F	5′ CATACTGGAAGGCATGGTGG 3′	MSP	63°C
	M	5′ CATGCTGGAAGGAATGGTGA 3′		
	R	5′ GTTTTGTGGAACCTGGAATAG 3′		

"T_a" refers to the PCR annealing temperature. Visualization of PCR products was by 1% agarose gel electrophoresis except where denoted by an "*" next to the mutation. These exceptions were visualized using standard SSCP techniques. See "Mutational Analysis" in the Experimental Procedures section. MSP is mutation-specific PCR.

mately 15/16 that of WT current, suggesting that one mutant D71V subunit is sufficient to eliminate channel function. Coexpression of WT and R218W Kir2.1 also induced inwardly rectifying K$^+$ currents (see Supplemental Figure S1 on *Cell* website), although the magnitude of current reduction was not as much as that seen with D71V subunits (Figure 6). Current amplitudes at −150 mV were −7.14 ± 1.18 μA, −3.80 ± 0.66 μA, −1.65 ± 0.13 μA, and −0.14 ± 0.01 μA for WT Kir2.1 (23 ng/oocyte), 1/2 WT (11.5 ng/oocyte), coinjected WT and R218W, and R218W alone, respectively. These findings demonstrate that D71V and R218W subunits coassemble with WT Kir2.1 subunits and cause variable degrees of dominant negative suppression of channel function.

Discussion

Mutations in Kir2.1 cause the phenotypes of Andersen's syndrome, including periodic paralysis, cardiac arrhythmias, and dysmorphic features. This conclusion is based on several findings. (1) An Andersen's gene is located on chromosome 17q23 near *KCNJ2*. (2) We identified a mutation D71V cosegregating with the Andersen's phenotype in kindred 4415. (3) An additional eight mutations were identified in unrelated Andersen's probands. All mutations occur at highly conserved amino acids and include deletion, substitution, and de novo mutations. None of the missense mutations were seen in over 100 unaffected, unrelated individuals. (4) Finally, electrophysio-
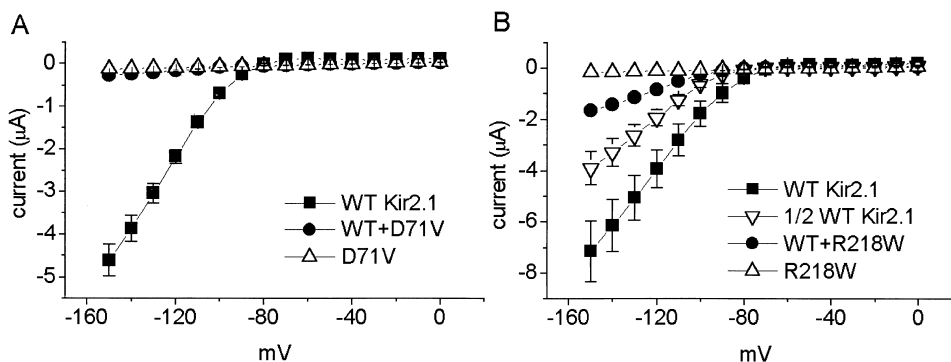

Figure 6. Functional Effects of D71V and R218W *KCNJ2* Mutations

(A) Instantaneous current-voltage relationships for oocytes injected with WT (filled squares), D71V (open triangles), and coinjected WT and D71V (filled circles) *KCNJ2*. Currents were elicited by step depolarizations from −150 to 0 mV, from a holding potential of −70 mV. No detectable K$^+$ currents were observed following injection of D71V alone, while coinjection of WT and D71V induced small, inwardly rectifying K$^+$ currents.

(B) Instantaneous current-voltage relationships for oocytes injected with WT (filled squares), 1/2 WT (down triangles), R218W (up triangles), and coinjected WT and R218W (filled circles) *KCNJ2*. Injection of R218W alone failed to induce detectable K$^+$ currents. Oocytes were injected with 23 ng total cRNA, with the exception of 1/2 WT that was injected with 11.5 ng WT cRNA. Currents induced by injection of 11.5 ng WT were approximately one-half that induced by 23 ng WT Kir2.1. Data represents mean ± SEM, n = 8–10 oocytes each group.

logical data on two of the mutations (D71V and R218W) show that these mutations cause a dominant negative effect on channel function when coexpressed with wild-type subunits as assayed by voltage-clamp experiments.

Kir2.1

Kir channels contribute to cell excitability and resting membrane potential in excitable tissues including heart, brain, and skeletal muscle (Doupnik et al., 1995; Jan and Jan, 1997; Nichols and Lopatin, 1997). Kir channels consist of an intracellular N-terminal domain, two transmembrane segments (M1 and M2) flanking a pore region, and an intracellular C-terminal segment (Figure 3). Kir subunits are believed to form either homo- or heterotetramers (Yang et al., 1995).

Kir2.1 is expressed predominantly in heart, brain, and skeletal muscle (Kubo et al., 1993; Raab-Graham et al., 1994). The function of Kir2.1 has been studied primarily in the heart. It is classified as a strong inward rectifier, which means that almost no current passes through these channels at potentials positive to −40 mV. Thus, strong inward rectification prevents excess loss of K$^+$ during the plateau phase of the cardiac action potential, but allows outward K$^+$ flux during terminal repolarization and diastolic phases of the action potential (Sanguinetti and Tristani-Firouzi, 2000). Much less is known about the role of Kir2.1 in other tissues such as the brain and skeletal muscle. It is likely that the role of Kir2.1 in skeletal muscle and neurons is similar to its role in the heart by controlling the resting membrane potential and the terminal repolarization phase of the action potential. Interestingly, there is some evidence suggesting that Kir2.1 has additional functional significance besides modulating the action potential of neurons and myocytes. Kir2.1 knockout mice have a complete cleft of the secondary palate and a slight narrowing of the maxilla (Zaritsky et al., 2000). In the rat, Kir2.1 mRNA is present by embryonic day 12 in bone-associated structure of the head, limb, and body (Karschin and Karschin, 1997).

Andersen's Syndrome Mutations Involve Residues in Important Functional Domains

Several lines of evidence suggest that all of the mutations we identified could have functional consequences for Kir2.1. First, all of the identified mutations involve residues that are very highly conserved across all families of the Kir subunits. Second, several mutations lie in functionally important domains such as the pore region containing the GYG K$^+$ selectivity filter. Finally, several mutations in Kir1.1 causing Bartter's syndrome, a renal disorder involving salt-wasting, hypokalemia, and metabolic acidosis, are in the same residue or similar functional domains as our mutations (Derst et al., 1997; Simon et al., 1996). For instance, D74Y in Bartter's syndrome is in the equivalent residue of our D71V mutation.

Dominant Negative Effects on Kir2.1 Function Have Consequences for Cardiac and Skeletal Muscle Excitability

LQTS is a disorder of cardiac myocellular repolarization manifested by prolongation of the interval between the onset of ventricular depolarization (QRS complex) and termination of ventricular repolarization (end of the T wave).

Dominantly inherited LQTS is due to mutations in the cardiac Na$^+$ channel (*SCN5A*) (Wang et al., 1995) or mutations in subunits encoding the cardiac delayed rectifier K$^+$ channels (*HERG*, *KCNQ1*, and *KCNE1*) (Curran et al., 1995; Sanguinetti et al., 1995; Splawski et al., 1997; Wang et al., 1996). The common pathophysiological feature of LQTS is prolongation of the cardiac action potential due to either enhanced depolarizing current (Na$^+$ channel mutations) or reduced repolarizing current (K$^+$ channel mutations). This study identifies *KCNJ2* as an LQTS gene and underscores the importance of Kir2.1 in modulating cardiac excitability. Kir2.1 was postulated to play an important, but not exclusive, role in generation of the cardiac inward rectifier current (I$_{K1}$) (Nakamura et al., 1998; Wible et al., 1995). I$_{K1}$ contributes significant repolarizing current during the terminal phase of the cardiac action potential and serves as the primary conductance controlling the diastolic resting membrane potential (E$_m$) in atrial and ventricular myocytes (Sanguinetti and Tristani-Firouzi, 2000). Reduction of Kir2.1 function would be expected to prolong the cardiac action potential and QT interval in affected individuals by reducing the amount of repolarizing current during the terminal phase. Action potential prolongation is a prerequisite for early afterdepolarizations (EADs), a presumptive trigger for ventricular tachycardia (January and Riddle, 1989). Whether mutations in Kir2.1 alter the diastolic E$_m$ in cardiomyocytes is not known.

Kir channels play an important, albeit secondary, role in controlling resting E$_m$ in skeletal muscle (Horowicz and Spalding, 1994). While Kir2.1, 2.2, and 2.4 are expressed in skeletal muscle, the relative contribution of each subfamily member is not known (Kubo et al., 1993; Takahashi et al., 1994; Topert et al., 1998). Our data demonstrate the importance of Kir2.1 in modulating skeletal muscle excitability. Mutations in Kir2.1 may sufficiently reduce the resting K$^+$ conductance such that E$_m$ is shifted in the depolarized direction, toward the equilibrium potential of chloride, the primary resting conductance in skeletal muscle (Horowicz and Spalding, 1994). Depolarization of the cell membrane would inactivate Na$^+$ channels, making them unavailable for initiation and propagation of action potentials. This model is consistent with a common feature in hypokalemic periodic paralysis (HypoPP), that is, reduced Na$^+$ current (Jurkat-Rott et al., 2000; Ruff, 2000; Ruff and Cannon, 2000). In individuals with HypoKPP caused by mutations in the L-type Ca^{2+} channel (Ptacek et al., 1994; Fouad et al., 1997), reduced availability of Na$^+$ channels is postulated to be linked to reduced activity of another Kir channel, K$_{ATP}$ (Ruff, 1999; Tricarico et al., 1999).

Recently, Abbott et al. (2001) described a missense mutation in *KCNE3* in two families with hyperkalemic periodic paralysis (HyperPP). *KCNE3* encodes the MinK-related peptide 2 (MiRP2) which coassembles with a voltage-gated K$^+$ channel, Kv3.4, to form a channel complex that contributes to resting E$_m$ in cultured skeletal muscle cells (Abbott et al., 2001). Mutant MiRP2-Kv3.4 complexes conduct less outward current than wild-type complexes, resulting in membrane depolarization and altered skeletal muscle excitability. Thus, our data and that of others (Ruff 1999; Tricarico et al., 1999; Abbott et al., 2001) support the importance of K$^+$ channels in modulating skeletal muscle excitability.

Kir2.1 in Development

The importance of ion channels in the function of muscle, heart, and brain is indisputable as evidenced by the plethora of mutations found associated with periodic paralysis, LQT, and epilepsy. However, an intriguing new niche for ion channels in development has just begun to be recognized, especially with the characterization of the *weaver* mouse. A mutation in the pore region of the G protein-coupled inward rectifier potassium channel GIRK2 was found to be associated with the defects in neural development of *weaver* (Patil et al., 1995).

Our findings in AS and Kir2.1 support the link between this ion channel and developmental processes. Andersen's syndrome and the Kir2.1 knockout mouse both provide evidence that Kir2.1 might play a previously unrecognized role in this process. Developmental characteristics of the Kir2.1 knockout mouse, including narrowing of the maxilla and complete cleft of the secondary palate (Zaritsky et al., 2000), are a potential analogy to the facial dysmorphology seen in many Andersen's patients. A study of Kir2.1 mRNA expression in rat embryos at embryonic day 12 shows that Kir2.1 mRNA is associated with bone structures in the head, limbs, and body (Karschin and Karschin, 1997). Whether or not Kir2.1 is expressed early enough for craniofacial and other bone morphogenetic events is currently being investigated. These studies could enhance our understanding of the putative role that Kir2.1 plays in craniofacial and skeletal morphogenesis.

Variable Expressivity of Andersen's Syndrome

Andersen's syndrome is incompletely penetrant and variably expressed. Severity ranges from nonpenetrant gene carriers (4 of 28 affected individuals), 1 of 3 characteristics (5 of 28), 2 of 3 characteristics (6 of 28), to severely affected with 3 out of 3 characteristics (13 of 28). Variation in expression of alleles, genetic background, or environmental factors could explain such pleiotropy. Because of the variable expressivity, it is likely that some Andersen's patients are diagnosed as LQTS or periodic paralysis patients instead.

Andersen's Syndrome Is Probably a Heterogeneous Disorder

While data presented in this paper demonstrate that *KCNJ2,* encoding Kir2.1, is an Andersen's syndrome gene, we also provide evidence suggesting genetic heterogeneity. No mutations in Kir2.1 coding sequence were detected in three Andersen families. One possibility is that these families have mutations in the regulatory regions of Kir2.1. An alternative hypothesis is that mutations in an unidentified partner or regulatory protein of Kir2.1 could also result in AS. Additional candidates for an AS gene are the other members of the Kir 2.x family (Kir2.2, 2.3, and 2.4). These other subunits have overlapping expression patterns with Kir2.1 in the brain, heart, and skeletal muscle (Morishige et al., 1994; Takahashi et al., 1994; Topert et al., 1998). Alternatively, mutations in completely unrelated genes could be responsible for Andersen's syndrome in the three unexplained families.

In summary, we have shown that mutations in the Kir2.1 gene, *KCNJ2,* are responsible for Andersen's syndrome. Our electrophysiological data on two of the mutations indicate that these amino acid changes result in dominant negative effects on Kir2.1 current. Additionally, our findings provide evidence that Kir2.1 plays an important role in skeletal and cardiac muscle function. A further in-depth electrophysiological study of all Kir2.1 mutations could provide additional insight into the structure and function of Kir2.1 in cells and could contribute to a better understanding of this syndrome. Finally, identification of Kir2.1 as the Andersen's syndrome gene extends the field of human channelopathies of the nervous system and heart, and dramatically underscores the similarities among these seemingly disparate episodic disorders.

Experimental Procedures

Identification and Phenotyping of Patients

There are no published clinical criteria for the diagnosis of Andersen's syndrome. The criteria used were based on the clinical data gathered from the three largest published case series (Canun et al., 1999; Sansone et al., 1997; Tawil et al., 1994). These criteria take into account the clinical observation that Andersen's syndrome shows variable penetrance. Individuals were classified as affected if two of three of the following criteria were met: clearcut episodes of muscle weakness, cardiac involvement, and dysmorphology. Muscle weakness was based on one of the following criteria: (1) a typical history of weakness with rest following exertion or prolonged rest, (2) an atypical history but with a documented physical exam during an attack demonstrating hyporeflexia with preserved sensation, (3) an atypical history without a documented exam but with unexplained intraictal serum hypo/hyperkalemia, or (4) an atypical history without a documented exam or serum potassium levels but with an abnormal exercise nerve conduction study (McManis et al., 1986). Cardiac involvement was determined by the presence of prolonged QTc on twelve-lead ECG according to standard criteria (Martin et al., 1995; Schwartz et al., 1993). Dysmorphology was noted if there was the presence of two or more of the following: (1) low set ears, (2) hypertelorism, (3) small mandible, (4) clinodactyly, or (5) syndactyly (persistent webbing between fingers). At risk individuals expressing one of the three major phenotypes of Andersen's syndrome were classified as "probably affected." Individuals were classified as unaffected if none of the criteria were fulfilled. One of the authors (R.T.), who was blinded to the results of the mutational analysis, reviewed the clinical information on each subject and confirmed their diagnostic classification.

Genotypic and Linkage Analyses

An automated genome-wide screen was performed on 15 individuals in kindred 4415 (Figure 2A) using the ABI marker index of 400 polymorphic markers. Markers were distributed across the genome at ~10–20 cM intervals. The fluorescently labeled markers were used to amplify genomic DNA in total reaction volumes of 20 μl in an MJR PTC-200 thermocycler (MJ Research). The products were visualized on an Applied Biosystems Model 373A and analyzed by the Genotyper peak-calling software. Pairwise linkage analysis was performed using the MLINK program of the LINKAGE package (Lathrop et al., 1985). Disease penetrance was set at 0.95 without a gender difference, and the normal and disease allele frequencies were set at 0.999 and 0.001, respectively.

Mapping

The region of chromosome 17q23 defined by the obligate recombinant boundaries of proximal marker D17S787 and distal marker D17S784 was examined in the mapviewer database (http://www.ncbi.nlm.nih.gov/genome/guide/). Candidate genes were selected based on their location within these boundaries as ascertained from the available physical map.

Mutational Analysis

The entire coding region of Kir2.1 was amplified (~1.6 kb) from genomic DNA in all individuals from kindred 4415. PCR primer sequence is as follows: F1 5′ CCAAAGCAGAAGCACTGGAG 3′ and

R1 5′ AATCAAATACCCAACCAAGGC 3′. 50 μl PCR reactions were performed on 100 ng of genomic DNA and 20 pmol of each F1 and R1 PCR primers using Clonetech's Advantage-GC cDNA polymerase and buffers. The GC-melt mix was used at a final concentration 1.0 mM, and reactions were cycled under the following protocol: 94°C–2 min, (94°C–10 s, 60°C–20 s, 68°C–2 min) × 45, 68°C–2 min and 30 s, 4°C–hold. These products were prepared using the Qiaquick PCR spin prep kit (Qiagen) and were sequenced using the following primers: F1, R1, F2 5′ GTGTTTGATGTGGCGAGTGG 3′ and R2 5′ ATTCCACTGTCAAACCCAAC 3′. Sequencing was performed by the Core facility at the University of Utah. Substitution mutations were checked in over 100 unaffected unrelated individuals either by SSCP analysis or by mutation-specific PCR analysis (See Table 1). For these individuals, genomic DNA was PCR amplified using the above protocol. These reactions were then diluted in 100 μl of water and served as templates with which to perform SSCP analysis or mutation-specific PCR (MSP) analysis. For SSCP and MSP, 2 μl of diluted PCR reaction was used as template DNA. SSCP was performed with 10 μl reactions as described previously (Ptacek et al., 1991). Products were electrophoresed according to Table 1 and visualized using standard techniques. MSP analysis was performed on five mutations that could not be visualized using SSCP. Individuals were PCR amplified using either the forward (F) and reverse (R) control primers or the forward mutant (M) primer and the reverse control primer. Products were electrophoresed side-by-side on a 1% agarose gel.

Mutagenesis of Kir2.1 and In Vitro Transcription
The human cardiac Kir2.1 was obtained in the plasmid pBluescript KS(−) as a gift from Carol Vandenberg (Raab-Graham et al., 1994). Mutagenic primers were designed incorporating the aberrant nucleotide with 9–10 base pairs on either side. Site-directed mutagenesis was performed using Promega's Gene-editor in vitro site-directed mutagenesis kit as recommended by the manufacturer. Putative mutagenic clones were sequenced by standard protocols in order to confirm the presence of the mutation.

Complementary RNAs (cRNA) for injection into oocytes were prepared with T7 Cap-Scribe (Boehringer Mannheim) following linearization of the expression construct with XhoI. cRNA was quantified by UV spectroscopy and gel electrophoresis.

Preparation and Injection of Oocytes
Isolation, maintenance, and injection of stage IV and V *Xenopus* oocytes with WT or mutant Kir2.1 cRNA were performed as described (Tristani-Firouzi and Sanguinetti, 1998). Briefly, *Xenopus* frogs were anesthetized by immersion in 0.2% tricaine for 15–30 min. Ovarian lobes were removed and digested with 2 mg/ml Type 1A collagenase (Sigma) in Ca²⁺-free ND96 solution containing (in mM): 96 NaCl, 2 KCl, 2 MgCl₂, 5 HEPES (pH 7.6). Stage IV and V oocytes were injected with cRNA (23–46 nl/oocyte), then cultured in Barth's solution supplemented with 50 μg/ml gentamycin and 1 mM pyruvate at 18°C. Barth's solution contained (in mM): 88 NaCl, 1 KCl, 0.4 CaCl₂, 0.33 Ca(NO₃)₂, 1 MgSO₄, 2.4 NaHCO₃, 10 HEPES (pH 7.4).

Two separate experiments were performed: in the first series, the ability of mutant subunits to form functional homomultimers was assessed by comparing currents induced by injection of WT or mutant Kir2.1 cRNA (23 ng/oocyte). In the second series, the ability of mutant subunits to coassemble with WT Kir2.1 was determined by comparing currents induced by WT Kir2.1 cRNA (23 ng/oocyte) to an equal mixture of WT and mutant Kir2.1 cRNA (11.5 ng/oocyte each). To assess endogenous currents, control oocytes were injected with 46 nl water.

Voltage Clamp of Oocytes
Whole-cell currents were recorded 3 days after cRNA injection using a GeneClamp 500 amplifier, a Pentium computer with a Digidata 1200 computer interface (Axon Instruments), and standard two-electrode voltage clamp techniques. Oocytes were bathed at room temperature (22–25°C) in a modified ND96 solution containing (in mM): 96 NaCl, 4 KCl, 2 MgCl₂, 0.1 CaCl₂, 5 HEPES (pH 7.6). Voltage clamp data were acquired and analyzed using pCLAMP 8 software (Axon Instruments). Currents were elicited by 200 ms pulses applied in 10

mV increments to potentials ranging from −150 to 0 mV from a holding potential of −70 mV.

Data Analysis
Digitized data were analyzed offline using pCLAMP8 and ORIGIN 6 (Microcal Inc.) software. Peak instantaneous current was measured after settling of the capacitance transient. Data are expressed as mean ± SE (n = number of oocytes).

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