Corticosteroid-exacerbated symptoms in an Andersen’s syndrome kindred

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Periodic paralysis, cardiac arrhythmia and bone features are the hallmark of Andersen’s syndrome (AS), a rare disorder caused by mutations in the KCNJ2 gene that encodes for the inward rectifier K⁺-channel Kir2.1. Rest following strenuous physical activity, carbohydrate ingestion, emotional stress and exposure to cold are the precipitating triggers. Most of the mutations act in a dominant-negative fashion, either through a trafficking dysfunction or through Kir2.1-phosphatidyl inositol bisphosphate binding defect. We have identified two families that were diagnosed with periodic paralysis and cardiac abnormalities, but only discrete development features. The proband in one of the two families reported having his symptoms occurring twice within the day following corticosteroids ingestion, and alleviated after stopping the corticosteroid treatment. Electromyographic evaluations pointed out to a typical hypokalemic periodic paralysis pattern. Molecular screening of the KCNJ2 gene identified two mutations leading to C54F and T305P substitutions in the Kir2.1 protein. Functional expression in mammalian cells revealed a loss-of-function of the mutated channels and a dominant-negative effect when both mutants and wild-type channels are present in the same cell. However, channel trafficking and assembly are not affected. Substitutions at these residues may interfere with phosphatidyl inositol bisphosphate binding to Kir2.1 channels. Sensitivity of our patients to multiple corticosteroid administrations shows that care must be taken in the use of such treatments in AS patients. Taken together, our data suggest the inclusion of the KCNJ2 gene in the molecular screening of patients with periodic paralysis, even when the classical AS dysmorphic features are not present.

INTRODUCTION

K⁺ channels are involved in a number of cellular processes that include repolarization, setting rest membrane potential and maintaining the electrolyte balance. Inward rectifier K⁺ channels are widely expressed in human tissues and are involved in many aspects of cell functions including excitability and differentiation. In excitable and non-excitable tissues, the Kir family has been shown to contribute to the resting of membrane potential, pacing of neuronal and cardiac myocytes, regulation of insulin secretion and renal transport (1). The Kir2 subfamily exhibits a strong inward rectification because of polyamines and Mg²⁺ block (2–5). The peptide consists of 428 amino acid residues that are predicted to have a secondary structure with an N terminus, followed by two membrane-spanning domains (M1 and M2) and a C-terminal part. The Kir2.1 inward rectifier channel may assemble as a homo- or hetero-tetramer to fashion a complex pore-forming protein (6). The Kir2.1 protein is encoded by the KCNJ2 gene, which has been associated with Andersen’s syndrome (AS), short QT syndrome and atrial fibrillation (7–9). Over 20 KCNJ2 mutations have been described in many AS kindreds (7,10–18). AS patients share many clinical phenotypes that include periodic paralysis.

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RESULTS

Electromyography

Electromyographic tests were performed in proband 1 (Fig. 1). The long exercise test (5-min duration) revealed a drastic and prolonged decrement in compound muscle action potential (CMAP) amplitude of the exercised muscle, beginning 10 min after exercise completion (Fig. 2) and worsening during the post-exercise rest (nadir: $-69\%$ reached 40 min after the end of the exercise). There was neither increment in the CMAP amplitude during the first seconds immediately after long exercise nor CMAP change after short exercise. These results were typical of the electromyographic (EMG) signs of abnormal, decreased muscle excitability described in HypoPP because of $\text{CACNA1S}$ mutations, $\text{SCN4A}$ mutations (21) or $\text{KCNJ2}$ mutations (16,22). As first molecular analyses failed to reveal the most recurrent $\text{CACNA1S}$ and $\text{SCN4A}$ mutations responsible for HypoPP, EMG pattern suggested searching for $\text{KCNJ2}$ mutations, which turned out to be positive.

cardiac arrhythmia and bone malformation as has been described in the first AS family (19). These diagnostic criteria have recently been extended to include skeletal and dental aspects (17,20). In AS, episodes of periodic paralysis are usually triggered by rest post-exercise, stress and carbohydrate intake. The precipitating factors reported in AS patients are quite similar to those reported for patients with other forms of periodic paralyses. However, corticosteroid intake has not been reported as a precipitating trigger in any of the AS patients. Here, we report two independent families carrying two novel $\text{KCNJ2}$ mutations. One of the identified AS families is of a particular interest because the proband has reported corticosteroids as being the trigger for two major attacks of periodic paralysis, that led to the diagnosis. We have successfully developed an electromyographic test on patients with discrete phenotype that can provide valuable information towards patient diagnosis. In addition, we carried out functional characterization of the two mutations to understand how they underlay the disease in the two families.

Novel Kir2.1 mutations at residues C54 and T305

Using direct sequencing of the solely known AS gene, we identified two novel mutations in two unrelated families with AS phenotype. Both probands were heterozygous for each mutation. Mutation G489T found in family 1 (the proband, the brother and the father) yield to C54F mutation in the kir2.1 protein, and is located in a well-conserved region of the N terminus. Mutation A1141C changed the threonine residue at position 305 to proline (T305P), located in the C-terminal part of the protein and is conserved as well among inward rectifier $K^+$-channel family (Fig. 3), suggesting an important role of these residues in channel function.

Dominant-negative pattern

$\text{KCNJ2}$ wild-type (WT) as well as G489T and A1141C mutants were subcloned into the pXOOM mammalian expression vector, and expressed in COS-7 cells. This vector is a bi-cistronic vector expressing $\text{KCNJ2}$ genes in the first site and enhanced green fluorescent protein (EGFP) in the second site, allowing the identification of positively transfected cells. Whole-cell patch-clamp technique was employed to monitor currents in transfected cells. As shown in Figure 4, WT channels showed normal inward rectifier currents. However, we were unable to record any current from both type of cells transfected with either kir2.1-C54F or kir2.1-T305P.

As both patients were heterozygous for the mutations, and because most of the AS mutants had a dominant-negative effects, we set to test the effect of co-expression of WT and mutants in the same cells. To make sure that both WT and mutant DNA are introduced into the same cell, we fused WT channels to DsRed monomer protein and mutant channels to EGFP. We and others have shown that fusion of fluorescent tags to kir2.1 protein did not affect either its biophysical properties nor its cell trafficking behavior (23). C54F-EGFP and...
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**DISCUSSION**

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already been involved in Kir2.1-PIP2 binding (K303, R312) and in channel trafficking (S314, Y315, L316). However, our confocal imaging data show normal trafficking of the mutated channels ruling out the involvement of this residue in any trafficking signaling on this channel. We hypothesize that T305 may destabilize Kir2.1-PIP2 binding leading to non-functional channels. This region of the channel, starting at residue 300 and ending at residue 315, seems to be a hotspot for AS mutations, as eight substitutions have been reported (G300V/D, V302M, E303K, T305P, T309I, R312C and ΔSY314-315). Pegan et al. (33) have recently resolved the structure of this region of the channel to 2.0 Å, and have shown that this region belongs to the G-loop that is involved in intra- and inter-subunit salt bridges that mainly affect channel gating.

Both mutations showed a loss-of-function when expressed alone and a dominant-negative behavior when co-expressed with the WT channels in mammalian cells, suggesting that the defective channels do co-assemble with WT channels to poison the protein complex, a behavior that is similar to that seen for most AS mutations. The amplitude of the current recorded in the presence of both WT and mutated channels suggests that only one defective subunit in the protein complex may be required for channel silencing. These data suggest that these two residues are not involved in channel assembly or trafficking. This conclusion is supported by confocal microscopy images showing normal trafficking of the mutated channels and a more likely normal assembly. In conclusion, we report clinical and functional characterizations of two novel AS mutations that have classical dominant-negative behavior. Our clinical data reveal that AS patients may have their symptoms triggered by corticosteroid intake. It would be of interest to determine the nature of corticosteroids that are involved, the mechanism underlying the exacerbation of the phenotype and the incidence of such phenomenon on a larger AS population.

Until then, care must be taken in using corticosteroid with patient harboring AS phenotypes. In addition, our study reveal that, in addition to AS triad phenotypes, KCNJ2 mutations may lead to periodic paralysis and abnormal EMG tests with mild or no AS dysmorphic features. This suggests to search for KCNJ2 mutations in patients with periodic paralysis, when molecular screening fails to reveal CACNA1S or SCN4A mutations.

**MATERIALS AND METHODS**

**Case histories**

Clinical studies were conducted after patients signed a consent form approved by the French and European Union bioethics law, and conformed with the Declaration of Helsinki. Patients were examined by one of the authors (S.G., D.M. or E.F.). Exams included electrocardiogram (ECG), EMG, CT scan and clinical electrophysiological study.

In family 1, the proband is a 19-year-old man with no family history (Fig. 1). He has a small chin, but no other noticeable dysmorphic features.

At the age of 15, the proband has experienced a restricted chin eruption that was first thought to be a herpes infection, and was later extended to the whole chin. He had then a local treatment associated with prednisolone, a corticosteroid-based anti-inflammatory drug. The first day of the corticosteroid intake, he reported important fatigue and difficulty to walk upstairs. When he woke up the next day, he was unable to sit down. At the hospital, he had hard time moving the upper limbs, and was unable to stand up. He complained from small muscle pain. The ECG showed extrasystoles, U waves and bigeminy. His blood exam revealed hypokalemia (2.2 mEq/L). His creatine phosphokinase (CPK) level was slightly over normal at 211 U/I (normal <174 U/I).
His symptoms disappeared 24 h later: he recovered almost all his movements, ECG was normal (with no extrasystolis), and kalemia returned to normal. During the following 4 weeks, he did not develop paralysis but he complained of muscle pain. No other corticosteroid side effect has been reported. His CPK level was as high as 997 U/l. He is under Diomax, Tenormine (β-blocker) and a potassium supplement. Since then, he had few periodic paralysis attacks, that were severe, and one of them was again triggered by corticosteroid therapy following an important urticaria of the upper and lower limbs. Beside corticosteroid intake, we were unable to determine other periodic paralysis triggering factors.

The proband in family 2 is a 23-year-old woman with no noticeable dysmorphic features. She reported having difficulties practicing sport at school at younger age. She described pain in lower limbs making it harder to walk during couple of days. Pain and weakness were not particularly triggered by exercise, rest after exercise, food or cold. She has full strength in upper limbs but permanent motor deficit in lower limbs. No muscle atrophy or myotonia was observed during her clinical examination. Muscle biopsy was normal. Clinical examination and muscle biopsy are in favor of a hypokalemic periodic paralysis pattern. ECG revealed ventricular arrhythmia, bidirectional tachycardia and extrasystolis. Family history shows cardiac arrhythmia in the mother, her aunt and grandmother (has a pacemaker), but a healthy brother (Fig. 1).

**Molecular diagnosis**

Patients' DNA was extracted from peripheral blood leukocytes and used as a template for screening and subcloning. Whole KCNJ2 open reading frame was PCR-amplified using pfu tag DNA polymerase (Promega, USA). PCR product was cloned into the pGEM-T vector (Promega, USA), and the clones were directly sequenced. Mutation-containing clones were PCR-amplified with primers flanked with restriction enzyme sequences: BamHI for cloning into the pXOOM vector, or EcoRI and SacII for cloning into the pEGFP vector. The latter allowed fusion of the EGFP to the C terminus of the Kir2.1 WT or mutant protein. Kir2.1 WT protein (WT human cardiac KCNJ2 was obtained in the plasmid pBluescript KS (-) as a gift from Dr Vandenberg, University of California San Francisco, USA) was also fused to DsRed monomer fluorescent protein using the same restriction enzymes.

The mutated fusion proteins were PCR-amplified using primers flanked with NheI and EcoRI sequences for sub-maximal stimulation of ulnar nerves at the wrist. The effects of two kinds of exercises were tested: (1) short exercise (10 s) of the left ADM muscle, with recording of the CMAP immediately after exercise cessation and then every 10 s for 1 min; (2) long exercise (5 min) of the right ADM muscle, with CMAP recording immediately after exercise completion, and then every minute for 5 min and every 5 min for 40 min. CMAP amplitude, duration and area were expressed as a percentage of the reference values measured before exercise. Changes in CMAP amplitude were compared to those observed in 30 healthy individuals.
sequent cloning into the first cloning site of the pIRES vector (Clontech) under CMV promoter. Kir2.1-DsRed fusion was amplified with primers flanked with NotI site and subcloned into the second cloning site to generate a plasmid allowing simultaneous expression of WT and mutant protein. All PCR products and final constructs were sequenced to ensure fidelity.

**Electrophysiology**

COS-7 cells were maintained in Dulbecco’s modified Eagle media supplemented with 10% fetal bovine serum and 100 μg/mL streptomycin, 100 U/mL penicillin, at 37°C in a humidified 5% CO2 atmosphere. COS-7 cells were transiently transfected by DEAE-Dextran precipitate method using 200 ng Kir2.1WT or mutant DNA per 35-mm culture dish. Currents were recorded 48 h after transfection.

Recordings were conducted in the whole-cell configuration (34) at room temperature (~22°C), using an EPC 10 amplifier (HEKA Electronic, Germany). The pipette solution contained (mM): 150 KCl, 0.5 MgCl2, 5 EGTA and 10 HEPES, pH 7.3. The bathing media were (mM): 150 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2 and 10 HEPES, pH 7.3. Pipette resistance was 1.5–4 MΩ. Membrane currents were elicited by depolarizations ranging from −120 to +40 mV, from a holding potential of −80 mV. Only cells with series resistance less than 5 MΩ were used for analysis. Data acquisition and analysis were performed using Patchmaster and Pulsefit (HEKA Electronic, Germany) and IgorPro (WaveMetrics, Inc., Oregon, USA) softwares.

**Confocal microscopy imaging**

COS-7 cells were transiently transfected with DNA encoding for EGFP, DsRed monomer (DsRed), Kir2.1-EGFP, Kir2.1-DsRed, Kir2.1C54F-EGFP, Kir2.1T305P-EGFP, Kir2.1-DsRed/Kir2.1-EGFP, Kir2.1-DsRed/Kir2.1C54F-EGFP and Kir2.1-DsRed/Kir2.1T305P-EGFP. COS-7 cells were cultured on a cover slip and transiently transfected for EGFP, DsRed monomer (DsRed), Kir2.1-EGFP, Kir2.1-DsRed/Kir2.1T305P-EGFP. COS-7 cells were maintained in Dulbecco’s modified Eagle media supplemented with 10% fetal bovine serum and 100 μg/mL streptomycin, 100 U/mL penicillin, at 37°C in a humidified 5% CO2 atmosphere. COS-7 cells were transiently transfected with DNA encoding Kir2.1-EGFP, Kir2.1-DsRed/Kir2.1-EGFP, Kir2.1-DsRed/Kir2.1C54F-EGFP and Kir2.1-DsRed/Kir2.1T305P-EGFP. Confocal microscopy imaging (Leica) images were used for analysis. Data acquisition and analysis were performed using Patchmaster and Pulsefit (HEKA Electronic, Germany) software.

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