EXPEDITED PUBLICATION

Sodium Channel Inactivation Defects Are Associated with Acetazolamide-Exacerbated Hypokalemic Periodic Paralysis

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A novel mutation in a family with hypokalemic periodic paralysis is described. The mutation R672S is located in the voltage sensor segment S4 of domain II in the *SCN4A* gene encoding the human skeletal muscle voltage-gated sodium channel. Functional expression of the R672S channels in human embryonic kidney 293 cells revealed a small but significant hyperpolarizing shift in the steady-state fast inactivation, and a dramatic enhancement in channel slow inactivation. These two defects are mainly due to a slow recovery of the mutant channels from fast and/or slow inactivation. Our data may help explain the mechanism underlying hypokalemic periodic paralysis and the patient's worsening from acetazolamide.

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Hypokalemic periodic paralysis (hypoKPP) is an autosomal dominant disorder characterized by severe muscle weakness accompanied by hypokalemia that may last several hours to days, and can be precipitated by rest after exercise, stress, fatigue, and carbohydrate ingestion. Acetazolamide prevents attacks of weakness in many patients. Mutations in the *CACN1AS* gene, encoding the dihydropyridine sensitive voltage-gated L-type calcium channel, are responsible for many hypoKPP.[1,2] Recently, genetic heterogeneity was observed among hypoKPP families, and the *SCN4A* gene, encoding the skeletal muscle sodium channel, was linked to hypoKPP.[3] The mechanism underlying this disease is not yet understood. A reduction in calcium channel conductance,[4–6] a sodium current reduction and an enhancement of slow sodium channel inactivation,[7] as well as a reduction of the ATP-sensitive potassium channel current,[8] were postulated to cause hypoKPP.

One common feature of the naturally occurring mutations causing hypoKPP is their location on the voltage sensor S4. Three mutations were identified on the calcium channel, and two mutations were identified on the sodium channel.[1–3,9] In the present study, we report a novel mutation occurring on DII-S4 of the skeletal muscle sodium channel found in a family with a history of hypoKPP in whom acetazolamide caused dramatic worsening of the disease.[10] We show that this mutation affects fast and slow sodium channel inactivation in a manner to produce paralysis in the patients harboring the R672S substitution.

Patients and Methods

Patients, Genetic Analysis, and Mutagenesis

Clinical studies were conducted after patients signed a consent form approved by the Institutional Review Board of the Utah Health Sciences Medical Center. Genomic DNA was extracted from peripheral blood leukocytes and amplified using polymerase chain reaction (PCR).[1] Single-stranded conformational polymorphism (SSCP) analysis was performed on amplified DNA.[11] Oligonucleotide primers used to amplify exon 5, 12, and 13 sequences encoding the S4 segments of domains I and II were (5′-3′): ACCCACCCCAGGCTCTGACA(Forward [F]), CAGGCTTTGCTGTGAGTTCT (Reverse [R]), CCTGGCCCTGGGCTTTTGTG (F), GTGGGAGTTGGGTGGGAGAC (R), CATTCACCCTTGCCCTCCCT (F), CGATGAGGAAGGAGTGGAAGA (R). The presence of the R672S mutation was confirmed by SSCP and by direct sequencing of patient DNA.

A mutagenic primer was designed incorporating the C2091A change (5′CTACGCTCCTTCAGTCTGCT3′). Site-directed mutagenesis was performed using Promega's Gene Editor in vitro site-directed mutagenesis kit as recommended by the manufacturer.

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Cell Culture and Electrophysiology

HEK 293 cells were grown and transfected.[11] The media was changed 15 hours after transfection, and recordings were conducted in the whole-cell configuration at room temperature (22°C).[12] Bathing solution was 140mM NaCl, 4mM $MgCl_2$, 2mM $CaCl_2$, and 10mM Na-Hepes at pH 7.3. The internal pipette solution was 130mM CsCl, 4mM $MgCl_2$, 2.5mM EGTA, 5mM NaCl, and 10mM Hepes at pH 7.3. (Values represent means ± SEM. Statistical data were obtained using student's *t* test.

Results

Evaluation of Patients

A family with hypoKPP was reported in 1981 because of paradoxical worsening with acetazolamide.[10] The 51-year-old man (Patient 1) first experienced episodic muscle weakness at age 15 years (Fig 1). Severe quadriparesis occurred every 1 to 3 months, usually on awakening. After age 20, attacks became less severe and tended to occur only after exercise. His 16-year old son (Patient 2) first experienced episodic weakness at age 13. Most attacks were of moderate severity, but in some attacks there was nearly total quadriplegia. Attacks occurred on awakening and were often precipitated by rest after exercise or by fever. His 22-year-old son (Patient 3) experienced rare attacks of weakness. The potassium level of Patient 3 was 2.6mEq/L during a spontaneous attack. No clinical or electrical myotonia was present in any of the 3 patients.

Intravenous (IV) glucose-insulin testing provoked weakness whereas oral potassium loading and cold provocation did not. Treatment with acetazolamide resulted in severe quadriparesis within 12 hours in Patient 1 on three occasions. A similar response was noted in Patients 2 and 3 within 48 to 60 hours. On triamterene (a potassium-sparing diuretic), all 3 patients improved and had only rare, slight attacks of weakness. Patient 4 had a single episode of severe weakness in childhood in the context of an acute gastrointestinal illness with vomiting. However, his IV glucose (90 g) and IV insulin (5 units) loading test was negative. Patient 4 does not now have symptoms, suggesting a variable expressivity of the mutation in this family.

Identification of Nucleotide Alterations in the Patient DNA

DNA from the patients was amplified using primers for exons 5, 12, and 13. An aberrant SSCP conformer was noted in the affected patients. Sequence analysis of the aberrant conformer revealed a C2091A mutation (data not shown) of the *SCN4A* gene that predicts an arginine 672 to serine change in the human skeletal muscle sodium channel α-subunit. This mutation was absent in 100 normal unrelated individuals.

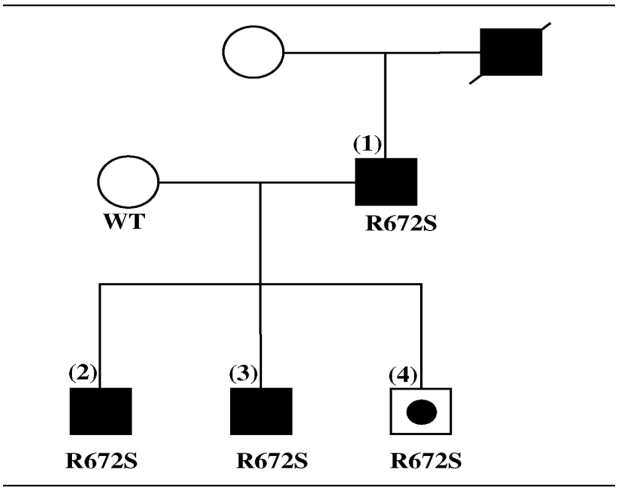

Fig 1. Pedigree of Kindred 2278 with hypoKPP. Patients are represented by filled symbols and unaffected individuals by open symbols (squares *for males*, circles *for females*). *Inner filled symbols represent unknown status. Patient 1 is the proband. Genotypes are as indicated.*

Functional Consequences of the R672S Mutation

To elucidate the mechanism by which mutation R672S may cause hypoKPP in Kindred 2278, the functional consequences were studied in HEK293 cells. Comparison of whole cell current traces from WT and the R672S mutation in Figure 2A shows that activation and fast inactivation kinetics are not affected. The activation curve for the mutant channels exhibited a very small shift but significant (WT, $V_{0.5} = -17.5 \pm 0.5$mV, n = 45; R672S, $V_{0.5} = -15.0 \pm 0.7$mV, n = 14; $p = 0.006$), with a slight change in the gating charge (WT, $z = 4.2 \pm 0.1$mV, n = 45; R672S, $z = 3.5 \pm 0.1$mV, n = 14; $p < 0.001$) (see Fig 2B). Moreover, R672S channels exhibited a significant ($p < 0.001$) change in the steady-state fast inactivation (WT, $V_{0.5} = -62.1 \pm 0.8$mV, n = 43; R672S, $V_{0.5} = -68.8 \pm 0.6$mV, n = 16) (see Fig 2B).

Because the mutant channels exhibited an enhancement in fast inactivation, we further investigated the channel fast inactivation parameters by testing their ability to recover from fast inactivation. At −80mV, a significant number of channels did not recover from fast inactivation in the mutant channels (see Fig 2C) compared with WT. However, mutant channel deactivation was similar to that of wild type suggesting that this mutation may not act through a voltage sensor immobilization to cause periodic paralysis (data not shown).

Studies of sodium channel mutations have shown that periodic paralysis can be precipitated by either an impairment or an enhancement in channel slow inactivation.[11,13,14] We hence investigated whether slow inactivation is affected in the R672S channels. As shown in Figure 3A, steady-state slow inactivation was en-

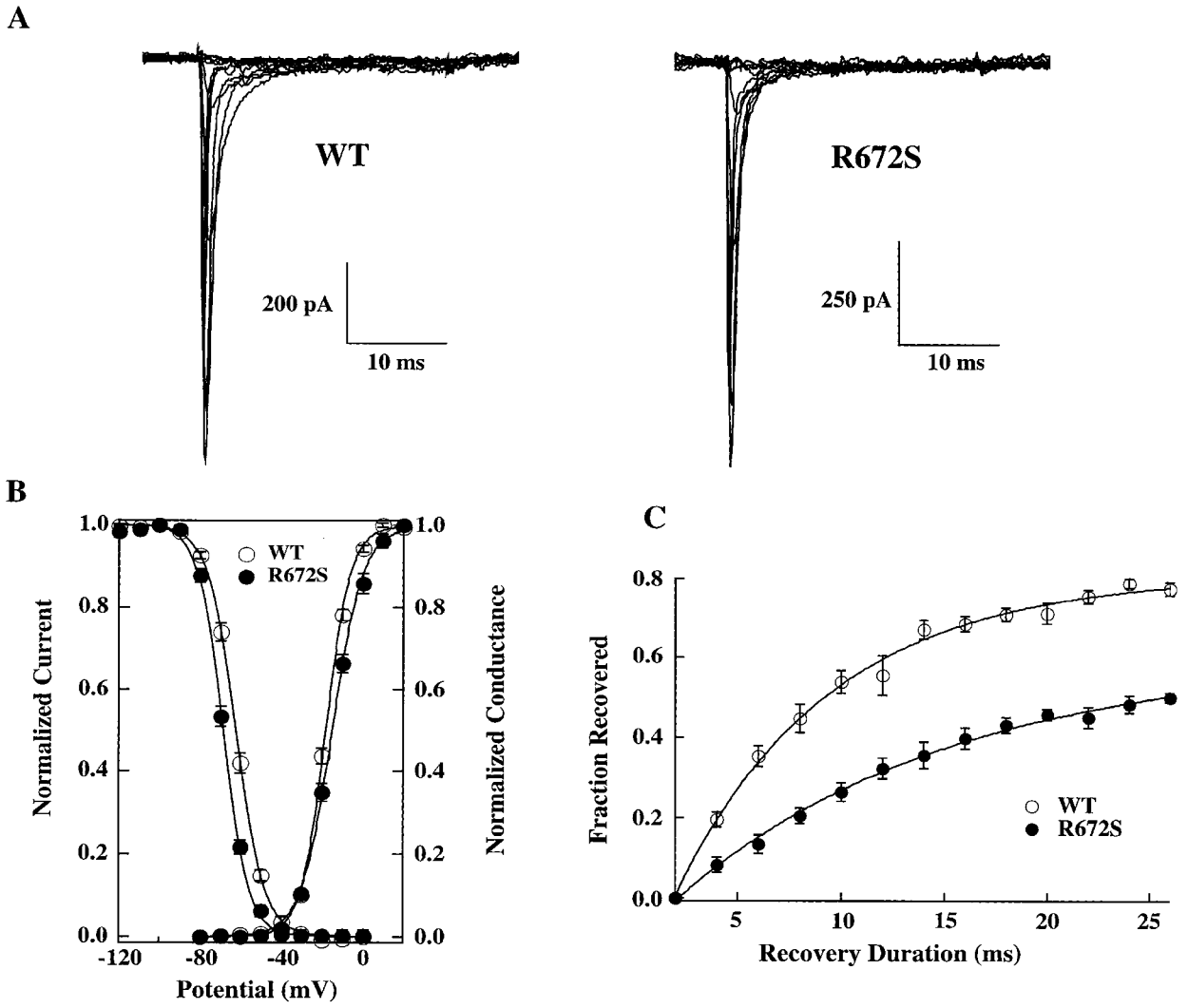

Fig 2. R672S affects fast inactivation. (A) Whole cell current traces from WT and R672S transfected cells. Cells were held at −120mV and depolarized for 25msec to potentials ranging from −80 to +40mV. (B) Steady-state activation was studied by measuring the peak sodium conductance during a 25msec test pulse to various test potentials, in 10mV increment, from −120mV holding voltage. To study steady-state fast inactivation, cells were held at prepulse potentials ranging from −120 to +20mV for 200msec, then subjected to a 0mV test pulse for 25msec.[11] (C) Recovery from fast inactivation was studied by pre-pulsing the cells to 0mV for 20msec to inactivate all of the current, then holding the cells to −80mV for increasing recovery durations prior to the test pulse to 0mV (20msec) to assay the fraction of current recovered.[11]

hanced in the R672S channels (WT, $s_{0.5} = -63.4 \pm 2.4$mV, n = 10; R672S, $s_{0.5} = -79.4 \pm 2.7$mV, n = 7). Only 50% of the channels were slow inactivated at −60mV, whereas about 90% of the mutant channels are slow inactivated at the same holding potential (see Fig 3A). The enhancement in slow inactivation was mainly due to a slow channel recovery from slow inactivation (see Fig 3B).

Discussion

hypoKPP is usually caused by mutations in the voltage gated calcium channel,[15] but other channels have subsequently been found to be involved in hypoKPP.[3,8] We here present a novel mutation, in the *SCN4A* gene, that co-segregates in a family with a history of hypoKPP. The R672S mutation is located in the segment S4 of domain II believed to be the voltage sensor.

Functional expression of the mutation revealed an enhancement of both fast and slow inactivation. This is represented by the negative shift of the steady state fast and slow inactivation. An enhancement in slow inactivation has already been reported with mutations responsible for periodic paralysis.[7,14,16] Slow inactivation is a process by which muscle cells may limit tissue damage by preventing long depolarizations of muscle by sodium channels. In the present study, the R672S channels have an enhanced slow inactivation that is due to a slower recovery from slow inactivation that

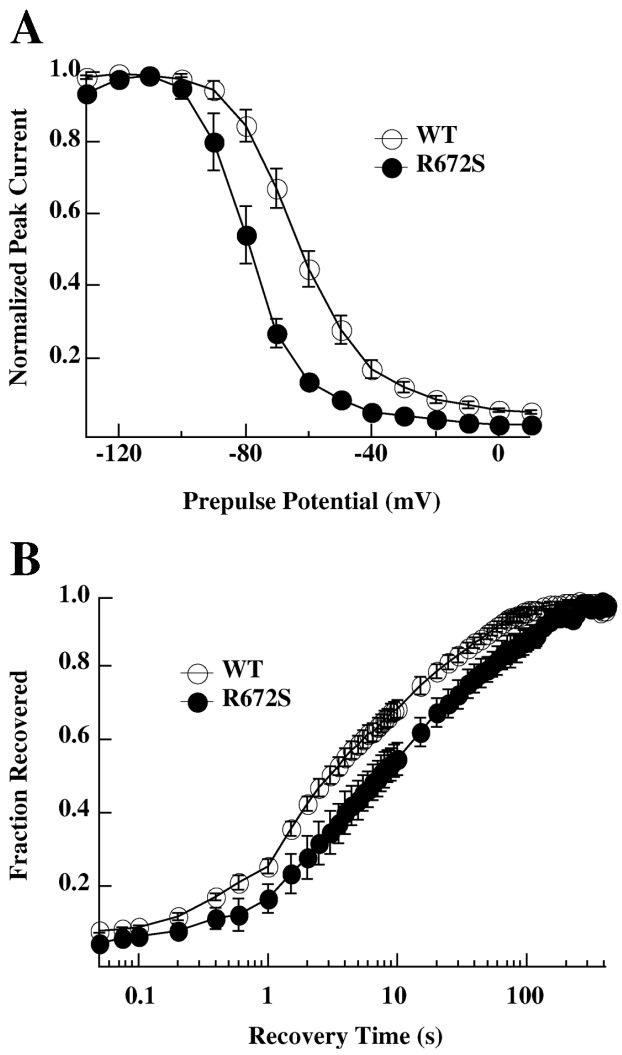

Fig 3. Slow inactivation is enhanced in R672S channels. (A) Steady-state slow inactivation was measured with 50-second conditioning pulses.[14] A 30ms recovery pulse to −100mV was used before each test pulse (20msec to −10mV) to remove fast inactivation. Steady-state slow inactivation data were fitted with a Boltzmann function. (B) Recovery from slow inactivation: cells were held at −20mV for at least 5 minutes. Recovery at −100mV was monitored.[14]

may result in long refractory periods leading to episodes of weakness. Our data corroborate a recent study of a mutation located in the same segment S4 (R669H) causing hypoKPP that also enhances slow inactivation[7] without known effect of acetazolamide.[3] Taken together, our data demonstrate that mutations in the DII-S4 may underlie the manifestation of hypoKPP by enhancing both fast and slow inactivation. This mutation may account for the worsening by acetazolamide.

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