Hypokalaemic periodic paralysis with a charge-retaining substitution in the voltage sensor

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BRAIN COMMUNICATIONS

Hypokalaemic periodic paralysis with a charge-retaining substitution in the voltage sensor

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Familial hypokalaemic periodic paralysis is a rare skeletal muscle disease caused by the dysregulation of sarcolemmal excitability. Hypokalaemic periodic paralysis is characterized by repeated episodes of paralytic attacks with hypokalaemia, and several variants in *CACNA1S* coding for $Ca_V1.1$ and *SCN4A* coding for $Na_V1.4$ have been established as causative mutations. Most of the mutations are substitutions to a non-charged residue, from the positively charged arginine (R) in transmembrane segment 4 (S4) of a voltage sensor in either $Ca_V1.1$ or $Na_V1.4$. Mutant channels have aberrant leak currents called 'gating pore currents', and the widely accepted consensus is that this current is the essential pathological mechanism that produces susceptibility to anomalous depolarization and failure of muscle excitability during a paralytic attack. Here, we have identified five hypokalaemic periodic paralysis cases from two different ethnic backgrounds, Japanese and French, with charge-preserving substitutions in S4 from arginine, R, to lysine, K. An R to K substitution has not previously been reported for any other hypokalaemic periodic paralysis families. One case is R219K in $Na_V1.4$, which is located at the first charge in S4 of Domain I. The other four cases all have R897K in $Ca_V1.1$, which is located at the first charge in S4 of Domain III. Gating pore currents were not detected in expression studies of $Ca_V1.1$-R897K. $Na_V1.4$-R219K mutant channels revealed a distinct, but small, gating pore current. Simulation studies indicated that the small-amplitude gating pore current conducted by $Na_V1.4$-R219K is not likely to be sufficient to be a risk factor for depolarization-induced paralytic attacks. Our rare cases with typical hypokalaemic periodic paralysis phenotypes do not fit the canonical view that the essential defect in hypokalaemic periodic paralysis mutant channels is the gating pore current and raise the possibility that hypokalaemic periodic paralysis pathogenesis might be heterogeneous and diverse.

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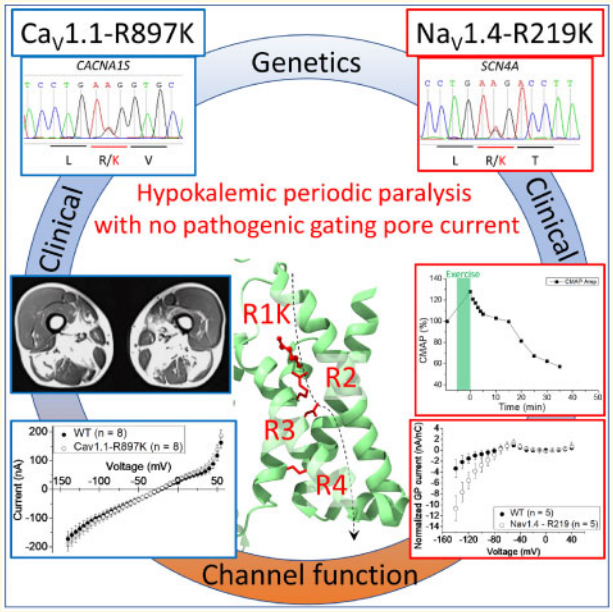
Abbreviations: CMAP = compound muscle action potential; HypoPP = hypokalaemic periodic paralysis; MRC = Medical Research Council; VSD = voltage sensor domain.

Graphical Abstract



Introduction

Familial hypokalaemic periodic paralysis (HypoPP) is a rare skeletal muscle disease caused by the dysregulation of sarcolemmal excitability (Cannon, 2015). HypoPP is characterized by repeated episodes of weakness in the setting of hypokalaemia, and several mutations in *CACNA1S* encoding $Ca_V1.1$ and *SCN4A* encoding $Na_V1.4$, have been identified as causative (Venance *et al.*, 2006). Most mutations are substitutions of a positively charged arginine (R) in the fourth transmembrane segment (S4) within a voltage sensor domain (VSD) of either $Ca_V1.1$ or $Na_V1.4$ (Cannon, 2010). The reason why mutations in two different channels can cause identical clinical manifestations had been a mystery for a long time. The explanation came when the biophysical experiments using Shaker K^+ channel and voltage-gated Na^+ channels demonstrated that missense mutations of arginine residues in the S4 segments create a leakage current, called the 'omega current' or 'gating pore current' (Starace and Bezanilla, 2004; Sokolov *et al.*, 2007). Subsequently, similar leak currents were detected in muscle fibres isolated from HypoPP mouse models with $Ca_V1.1$-R528H and $Na_V1.4$-R663H, S4 arginine to histidine knock-in mutations (Wu *et al.*, 2011, 2012). Computational models of fibre excitability show that although the gating pore leak is small (about 5% of the total resting conductance of the fibre), the current is sufficient to cause paradoxical depolarization of the resting potential in low extracellular K^+, which is the hallmark of HypoPP. This 'gating pore current theory' has been postulated to be the common pathogenic feature in HypoPP (Matthews *et al.*, 2009; Cannon, 2010). Interestingly, a mutation of the α2 subunit of the Na^+/K^+-ATPase encoded by *ATP1A2* has been recently reported to be responsible for HypoPP by producing anomalous leak current, which further strengthens the unifying pathomechanism (Sampedro Castañeda *et al.*, 2018).

For 19 of 20 reported HypoPP mutations, the genetic lesion causes substitution of arginine to a non-charged

residue in the S4 segment of a VSD. Functional expression studies have demonstrated gating pore currents in all 11 HypoPP R/X mutations in S4 segments of $Na_V1.4$ channels (Sokolov *et al.*, 2007; Struyk and Cannon, 2007; Struyk *et al.*, 2008; Francis *et al.*, 2011; Bayless-Edwards *et al.*, 2018) and all 4 of the S4 R/X mutants tested in $Ca_V1.1$ (Wu *et al.*, 2012, 2018b; Fuster *et al.*, 2017*a*). For the one exception, p. Val876Glu in the S3 segment of domain III in $Ca_V1.1$, expression studies also detected a gating pore current (Fuster *et al.*, 2017*a*). All available data are consistent with the notion that the gating pore current, usually as the result of a non-charged R/X missense mutation in the S4 segment of a VSD, is the essential anomaly that produces susceptibility to episodic loss of fibre excitability during attacks of weakness in HypoPP. Moreover, from the biophysical point of view on the structure–function relations for the VSDs in $Na_V1.4$ or $Ca_V1.1$, it is plausible that the loss of a positively charged residue might be necessary for the generation of gating pore currents, by disrupting the interactions with the counter negatively charged residues, glutamate (E) or aspartate (D), in S2 and S3 (Tao *et al.*, 2010; Pan *et al.*, 2018).

Here, we report that five patients with typical HypoPP clinical phenotypes from four pedigrees (two Japanese and two French), harbouring missense from arginine to lysine (R/K) mutations, which preserves the positive charge in the voltage sensor in either $Na_V1.4$ or $Ca_V1.1$. We detected a gating pore current for the R219K mutation in $Na_V1.4$, albeit with a very small amplitude that was about 1/17th of that seen for other non-charge preserving R/X HypoPP mutations. Conversely, we were unable to detect a gating pore current for the R897K mutation in $Ca_V1.1$. Our results suggest that alternative mechanisms, besides the canonical gating pore current, may also be a cause of intermittent loss of fibre excitability in HypoPP.

Materials and methods

Patient consent and genetic analysis

We reported clinical information of Japanese patients under the approval of the ethical committee in Osaka university medical hospital. We conducted the genetic analysis after obtaining written form of the informed consent which has been approved by the ethical committee in Osaka university graduate school of medicine. Genetic diagnosis in French patients was performed in accordance with French Bioethics Laws.

Genomic DNA was extracted from the patients' blood lymphocytes. The region comprising all exons of *SCN4A*, *CACNA1S* and *KCNJ2* gene were amplified by PCR, and the purified fragments were sequenced using an automated DNA sequencer (Big Dye Terminator v 3.1 and ABI310; PE Applied Biosystems, Foster City, CA, USA).

Molecular biology

The α and $\beta1$ subunits of the rat skeletal muscle voltage-gated sodium channel ($rNa_V1.4$) were cloned into pBSTA vectors for expression studies in *Xenopus* oocytes. For patch-clamp experiments using HEK293t cells, the human skeletal muscle $Na_V1.4$ channel ($hNa_V1.4$) and $\beta1$ subunit were cloned into the mammalian expression construct, pRc/CMV. The R291K missense mutation of $Na_V1.4$ was introduced with the site-directed mutagenesis kit (KOD-Plus mutagenesis kit; TOYOBO, Osaka, Japan) and verified by Sanger sequencing.

The human α_{1S} subunit of the calcium channel ($hCa_V1.1$) was co-expressed with rat $\alpha_2\delta$, rabbit β_1 and mouse Stac3, as previously described (Wu *et al.*, 2018*b*). The $Ca_V1.1$, Stac3 and β_1 constructs were subcloned into an oocyte-optimized expression vector, pGEMHE (Liman *et al.*, 1992) and the $\alpha_2\delta$ cDNA was subcloned into pcDNA3. Site-directed mutagenesis of $Ca_V1.1$ to create $Ca_V1.1$-R897K was performed with the QuikChange II mutagenesis kit (Agilent) and verified by sequencing the entire cDNA insert and flanking regions of pGEMHE.

Expression of $rNa_V1.4$, $rNa_V\beta1$ and $hCa_V1.1$ in *Xenopus* oocytes

For expression studies of $rNa_V1.4$ in oocytes, cRNAs for the α and $\beta1$ subunits were transcribed *in vitro* using the T7 mMESSAGE cRNA kit (Ambion). Freshly isolated oocytes from *Xenopus laevis* were injected with 75 ng of a 1:1 molar ratio of cRNAs and maintained in standard oocyte saline solution: 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 200 mg/L sodium pyruvate, pH 7.4, for 1–4 days at 18°C. Expression studies of $Ca_V1.1$ were performed similarly, except 100 ng of the cRNA mixture ($Ca_V1.1$, $\alpha_2\delta$, β_1 and Stac3) was injected and oocytes were stored in 0.5× Leibovitz's L-15 medium (HyClone) supplemented with 1% horse serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 100 µg/ml amikacin. All experiments were performed within the guidelines established by the Institutional Animal Care and Use Committees at the University of Chicago and the University of California, Los Angeles.

Expression of $hNa_V1.4$ and $h\beta1$ in HEK293t cells

Expression studies of $hNa_V1.4$ in mammalian cells were performed by transient transfection in HEK293t cells as previously described (Takahashi and Cannon, 1999). Briefly, plasmid cDNAs encoding wild type and mutant human $Na_V1.4$ α-subunits (0.42 μg/35-mm dish), the human Nav channel $\beta1$-subunit (0.42 μg/35-mm dish) and a CD8 marker (0.08 μg/35-mm dish) were transfected by calcium phosphate precipitation.

Electrophysiology

Sodium ionic currents were measured using a conventional whole-cell patch-clamp technique as described previously (Takahashi and Cannon, 1999). Briefly, at 2–3 days after transfection, the Human embryonic kidney (HEK) cells were trypsinized briefly and passaged onto 12 mm diameter round glass coverslips for electrophysiological recording. Individual transfection-positive cells were identified by labelling with anti-CD8 antibody crosslinked to microbeads. Currents were recorded with an Axopatch 200B amplified (Molecular Devices, San Jose, CA, USA). The tip of the electrode was heat-polished, and the resistance was 1.8–2.5 MΩ. Series resistance was 2–6 MΩ, and 75–80% of the series resistance was compensated by the analog circuitry of the amplifier. Cells with peak currents of <1 or >10 nA upon step depolarization from −120 mV to −10 mV were excluded. The pipette (internal) solution consisted of (mM): 105 CsF, 35 NaCl, 10 ethylene-glycol tetraacetic acid and 10 Cs-HEPES (pH 7.4). The bath solution consisted of (mM): 140 NaCl, 4 KCl, 2 CaCl2, 1 MgCl2, 5 glucose and 10 Na-HEPES (pH 7.4).

Charge-displacement currents and gating pore currents were recorded using a cut-open oocyte voltage clamp setup (Stefani and Bezanilla, 1998). For charge-displacement measurements, the external solution contained 115 mM *N*-methylglucamine-methylsulfonate (MS), 10 mM HEPES, and 2 mM Ca-MS, pH 7.4. The internal solution contained 115 mM *N*-methylglucamine-methylsulfonate, 10 mM HEPES and 2 mM ethylene-glycol tetraacetic acid, pH 7.4. For recording $rNa_V1.4$ currents, oocytes were voltage-clamped at −100 mV for at least 5 min to remove slow inactivation. After measurements of gating currents, the solution in the chambers was replaced into the pair of solutions; the external solution contained 115 mM Na-MS, 10 mM HEPES and 2 mM Ca-MS, pH 7.4 and the internal solution contained 115 mM *N*-methylglucamine-methylsulfonate, 10 mM HEPES and 2 mM ethylene-glycol tetraacetic acid, pH 7.4. To block ionic currents, 10 μM tetrodotoxin was applied to the upper and guard chambers. After replacing solutions, the oocyte membrane was held at −100 mV, again, for at least 5 min, then the gating pore currents were recorded. For recording Ca^{2+} currents in oocytes expressing $hCa_V1.1$ channels, the external solution contained 96 mM Na-MS, 6 mM Ca^{2+} acetate, 10 mM HEPES, pH 7.0 with methanesulfonic acid. In some gating pore experiments, the Na-MS was reduced to 60 mM and the solution was supplemented with 60 mM guanidinium acetate. The 'early current' 3–4 ms after a test depolarization was measured to test for the presence of a gating pore current. This time window is after the charge-displacement current has settled, but too early for activation of the Ca^{2+} current through the pore, and thereby provides an optimal interval isolating the gating pore current (Wu *et al.*, 2018*b*). Blockers were not used to suppress the Ca^{2+} current to avoid the possibility of also blocking a gating pore current.

Data analysis

The currents from *Xenopus* oocytes were recorded with a PC44 board and digitized on the 16-bit A/D converter, and were sampled at 10 ms/point. The data acquisition program was developed in house. For gating currents measurements, linear leak and membrane capacitive currents were subtracted manually using currents obtained from a subtracting holding potential of +20 mV. All data were obtained at 20°C. Gating pore currents were obtained without any subtraction.

The ionic currents obtained from HEK293t cells were acquired by Digidata 1440 A (Molecular Devices, San Jose, CA, USA) and pClamp 10 software (Molecular devices, San Jose, CA, USA) was used for the data collection. Curve fitting was manually performed using Origin software (Microcal LLC, Northampton, MA, USA) as stated previously (Takahashi and Cannon, 1999).

Statistical analysis

All data indicate the mean values ± standard error of the mean. Statistical significance was determined in cases where *P*-values were less than 0.05 by unpaired *t*-test.

Data availability

The data which support the findings in this study are available from the corresponding authors when we receive the reasonable requests.

Results

Cases

Case 1

The proband is a 16-year-old Japanese boy. One day, the patient felt muscle pains after Judo class. On the next day, the weakness in upper limbs appeared, spreading to lower limbs on the following day. On the fourth day after the judo class, the patient was brought to the emergency department because of severe weakness in all limbs. Upon arrival, he had tetraparesis with a Medical Research Council (MRC) Grade 2/5 in upper limbs and the Grade 1/5 in the lower limbs. There is no family history of neuromuscular disorders. The laboratory data showed hypokalaemia (1.5 mmol/l) and hyperCKemia (603 U/l), but no abnormality in thyroid function or adrenal function. The ECG revealed abnormal U waves and prolonged Q-Tc, most likely due to hypokalaemia. Potassium chloride was administered intravenously, with

monitoring of ECG and serum potassium. After a few days of treatment, his tetraparesis recovered completely, and he showed no neurological deficit. In order to confirm a suspected diagnosis of HypoPP, clinical neurophysiological assessment was performed. Nerve conduction study showed no abnormalities. Needle electromyography did not reveal myotonic discharges or spontaneous activity in all muscles examined including *biceps brachii*, *first dorsalis interosseus*, *vastus lateralis* and *tibialis anterior* muscles. The compound muscle action potential (CMAP) during the short exercise test showed no apparent changes (Supplementary Fig. 1). The prolonged exercise test, performed after potassium chloride administration, revealed a marked decrement of the CMAP amplitude (−30%) compared to the reference before the exercise task (Fig. 1B) (Fournier *et al.*, 2004; Tan *et al.*, 2011). Genetic analysis using the DNA extracted from the patient's and both parents' lymphocytes was performed. The proband had a heterozygous substitution (c. 2690G>A) in *CACNA1S* gene resulting in p. R897K mutation in $Ca_V1.1$, which was not present in either parent. The clinical, CMAP exercise, and genetic data support a diagnosis of *de novo* HypoPP type 1, with R897K missense mutation of $Ca_V1.1$ (Fig. 1A).

Cases 2-1 and 2-2

The probands were two French brothers. The elder brother, Case 2-1, experience repeated episodes of tetraparesis with hypokalaemia since adolescence. He developed a myopathy at age 55, at which time a muscle biopsy showed vacuoles and tubular aggregates. The patient died from liver cancer. A clinical electrophysiological assessment was never performed. The younger brother, Case 2-2, showed repeated episodes of tetraparesis since age 16. Serum potassium concentration during the paralytic attack was measured only once, which showed around 1 mmol/l. He reported 3–4 attacks per year over a period of 10 years that were triggered by rest after strenuous exercise, alcohol or carbohydrates-rich meals. The prolonged exercise test at the age of 57 showed a significant decrement of CMAP (−31%) representing the pattern V according to Fournier's classification. The frequency of the paralytic attacks decreased with the administration of acetazolamide and potassium chloride supplementation. Beginning at age 59, permanent weakness of the pelvic girdle muscle was noticed. The patient complained of limitations with running, climbing stairs and getting up from the ground. The lower-extremity MRI scan performed at age 64 showed muscle atrophy, fatty changes and the short tau inversion recovery hyperintensity in the posterior compartment of the lower leg (thigh and calve) (Fig. 1C, Supplementary Fig. 2). The genetic analysis of the two brothers showed a heterozygous R897K mutation in *CACNA1S* gene. Their father also had similar symptoms. They had four sisters who were asymptomatic and never had genetic testing.

Case 3

The proband was a French boy who presented with a first episode of tetraparesis at age 20, following a football match. The blood potassium level measured during the paralytic attack was 2 mmol/l. He experienced episodes of tetraparesis once or twice a month for about 10 years. Triggering factors were rest after sustained exercise, cold weather and carbohydrate-rich meals. Treatment with potassium chloride supplementation and potassium-sparing diuretics (spironolactone) significantly reduced the frequency of the paralytic attacks. Since the age of 46, he developed progressive permanent weakness of the pelvic girdle with difficulties climbing stairs or rising from squatting positions. A CT scan showed paravertebral and pelvic muscle atrophy with fatty infiltration (Fig. 1D). The prolonged exercise test revealed a 25% decrement of the CMAP amplitude, whereas a 40% decrement is considered diagnostic of periodic paralysis (Fournier *et al.*, 2004; Tan *et al.*, 2011). The genetic analysis showed a heterozygous R897K mutation in *CACNA1S* gene. He has three unaffected siblings who have never been genetically tested, and there is no family history of neuromuscular disorders.

Case 4

The proband was a 36-year-old Japanese male with no family history of neuromuscular disorders. Since the age of 30, he experienced transient muscle weakness in the lower limbs, twice a month on average, but never visited the clinic. At the age of 31, he was transferred to the emergency department and hospitalized because of severe tetraparesis. The laboratory data, while still weak, showed hypokalaemia (2.1 mmol/l), hyperCKemia (541 U/L), normal thyroid function and normal adrenal function. The ECG showed low amplitude T wave in aVF and III, and U waves in V2–V5, most likely due to hypokalaemia. By giving potassium chloride, the hypokalaemia normalized, followed by the recovery of tetraparesis. At age 34, the patient experienced similar attacks of severe tetraparesis again and consulted a neurologist for the diagnosis. When he was evaluated in our outpatient clinic, there were no abnormal findings in the neurological examination. The needle electromyography examination did not reveal myotonic discharges, but the prolonged exercise test elicited a large decrement of CMAP amplitude, more than 40% (Fig. 1F) (Fournier *et al.*, 2004; Tan *et al.*, 2011). The genetic analysis revealed the heterozygous substitution (c.656G>A) in the *SCN4A* gene resulting in p. R219K in $Na_V1.4$ (Fig. 1E). The material from other family members was not available for genetic analysis.

In addition to the specific variants identified for each of the cases above, we performed comprehensive whole-exon screening in all five patients for genes associated with familial periodic paralysis, including *SCN4A* (NM_000334.4), *CACNA1S* (NM_000069.3) and *KCNJ2* (NM_000891.2). No other variants, except for known

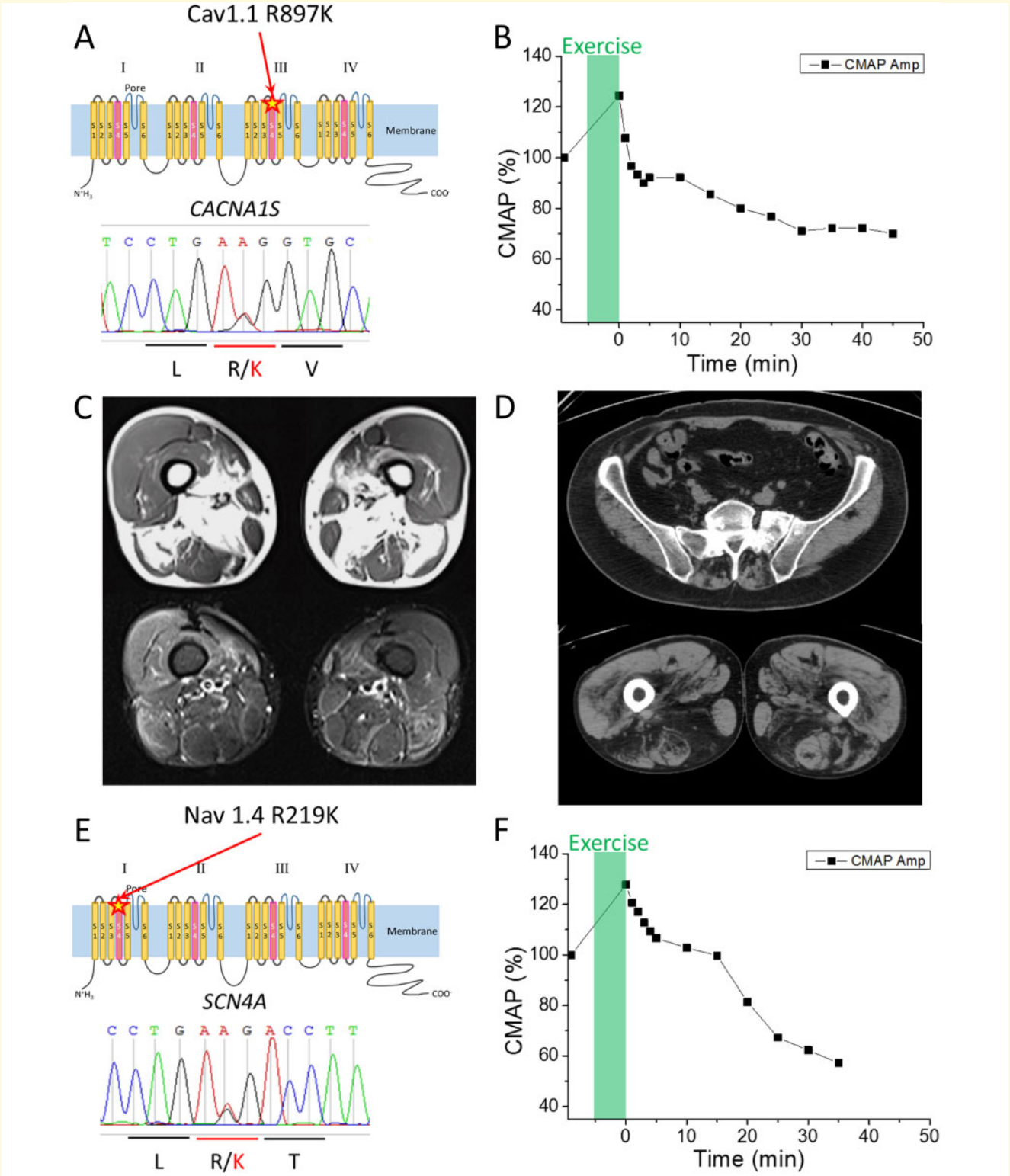


Figure 1 Clinical information of HypoPP patients with the R/K substitution in either *CACNA1S* or *SCN4A*. (**A**) Schematic representation of R897K location in $Ca_V1.1$ channel (upper panel) and the result of the Sanger sequencing obtained from Case 1. (**B**) Prolonged exercise test in Case 1 elicited an early increase and a late decrease of ~30% for the CMAP. (**C**) MRI images of skeletal muscles in Case 2-2. T1 weighted images (upper panel) and short tau inversion recovery images (lower panel) revealed fatty infiltration in *soleus* and *medial gastrocnemius* muscles. *Quadriceps*, *gracilis*, *sartorius*, *semi-membranosus* and *semi-tendinosus* muscles are grossly respected. (**D**) CT scan images of skeletal muscle in Case 3. CT scan image of lumbar portion (upper panel) showed low density area in paraspinal muscles, indicating fatty infiltration. CT scan image of thigh (lower panel) revealed low density area in bilateral hamstrings indicating fatty infiltration. (**E**) Schematic representation of the R219K location in $Na_V1.4$ (upper panel) and the results of Sanger sequencing obtained from Case 4. (**F**) Prolonged exercise test in Case 4 revealed a decrement of the CMAP by 40%, which supports a diagnosis of periodic paralysis.

single nucleotide polymorphisms, were detected. Neither p. R219K in $Na_V1.4$ nor p. R897K in $Ca_V1.1$ has been registered in the database of Tohoku Medical Megabank Organization (ToMMo), which has genotypes of 47 000 Japanese individuals (4.7KJPN), nor in the ExAC database. PolyPhen-2 (Adzhubei *et al.*, 2010) showed that R219K in $Na_V1.4$ is a 'probably damaging' mutation with a score of 1.000, whereas R897K in $Ca_V1.1$ is a 'benign' mutation with a score of 0.013. By Mutation Taster (Schwarz *et al.*, 2010), both R219K in $Na_V1.4$ and R897K in $Ca_V1.1$ are predicted as 'disease-causing' mutations. Sorting Intolerant From Tolerant (SIFT) (Sim *et al.*, 2012) displayed that both R219K in $Na_V1.4$ and R897K in $Ca_V1.1$ are 'intolerant'. Clinical information for all cases is summarized in Table 1.

Functional analysis of $Na_V1.4$-R219K and $Ca_V1.1$-R897K

Functional expression studies of ion channel mutations associated with periodic paralysis have revealed derangements in the voltage-dependent gating of $Na_V1.4$, and specifically for HypoPP, the presence of an anomalous gating pore current conducted through a 'leaky' VSD of $Na_V1.4$ or $Ca_V1.1$ HypoPP mutant channels (Sokolov *et al.*, 2007; Struyk and Cannon, 2007; Cannon, 2010, 2015; Mi *et al.*, 2014; Wu *et al.*, 2018b). The HypoPP mutations previously shown to support gating pore currents are almost all (19 of 20) missense mutations in the first or second arginine residues of S4 transmembrane segments (i.e. near the extracellular end). The charge-preserving R/K mutations reported herein are located at the first arginine of S4 in domain I of $Na_V1.4$ (R219K, Fig. 1E) and at the first arginine of S4 in domain III of $Ca_V1.1$ (R897K, Fig. 1A). We, therefore, measured Na^+ currents to characterize the voltage-dependence of gating for $Na_V1.4$ WT and R219K channels and tested for the presence of anomalous gating pore currents in $Na_V1.4$-R219K and $Ca_V1.1$-R897K by measuring currents at hyperpolarized potentials in the presence of pore blockers.

Voltage-dependence of gating was not disrupted by the $Na_V1.4$-R219K mutation

Sodium currents were measured from HEK293t cells transiently transfected with plasmids encoding WT or R219K $Na_V1.4$ plus the axillary $\beta1$ subunit. Expression of WT and R219K mutant channels was comparable with Na^+ current densities of -394.9 ± 104.0 A/F for WT ($n = 7$) and -306.5 ± 68.0 A/F for R219K ($n = 8$) based on the peak current at a test potential of -10 mV. The voltage-dependence of activation was indistinguishable for WT and R219K channels, as shown by the overlapping data in the conductance–voltage relation (Fig. 2A

Table 1 Clinical information summary

Case	1	2		3	4
Pedigree (race)	(Japanese)	(French)		(French)	(Japanese)
		2-1	2-2		
Sex	Male	Male	Male	Male	Male
Genetic diagnosis	R897K in *CACNA1S*				R219K in *SCN4A*
Onset	16 years old	Adolescence	16 years old	20 years old	30 years old
Family history	None (*de novo*)	+ (brothers and their father)		None	None
LET	+(30% decrement)	N/A	+(31% decrement)	+(25% decrement)	+(43% decrement)
Serum potassium during attack	Hypokalaemia (1.5 mmol/l)	Hypokalaemia	Hypokalaemia	Hypokalaemia	Hypokalaemia (2.1 mmol/l)
Other features	HyperCKemia(603 U/l) during PP attack	Myopathy (+) (vacuoles and tubular aggregates),Died of liver cancer	Acetazolamide effective	Myopathy (+)	HyperCKemia (541 U/l) during PP attack

LET indicates the prolonged exercise test.

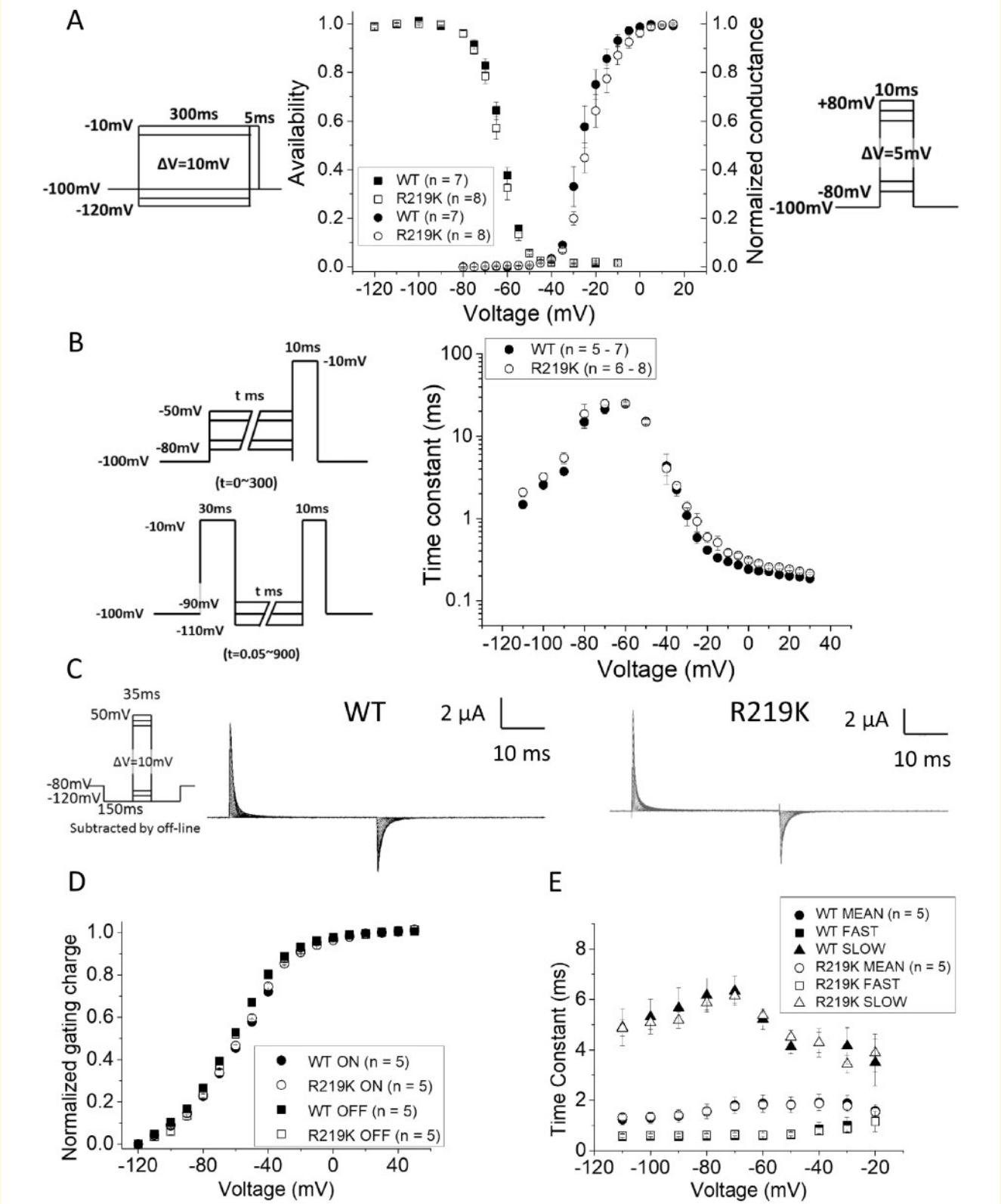


Figure 2 The functional analysis of $Na_V1.4$-R219K gating. (**A**) The voltage dependent conductance (G–V relationship) and the voltage dependent steady-state fast inactivation (V-hinf) in wild type $Na_V1.4$ ($n = 7$) and $Na_V1.4$-R219K ($n = 8$) are shown. The pulse protocols used are shown as insets, in the right for G-V and in the left for V-hinf. (**B**) Voltage dependent time constants for fast inactivation (Tau–V relationship) in wild type NaV1.4 ($n = 5$–7) and $Na_V1.4$-R219K ($n = 6$–8) are shown. The left upper panel indicates the two-pulse protocol for the entry to fast inactivation from −80 mV to −50 mV, and the left lower for the recovery from fast inactivation from −110 mV to −90 mV, respectively. Error bars indicate standard error of the mean. (**C**) Representative charge-displacement currents from the wild type of $Na_V1.4$ (WT) and $Na_V1.4$ with

(continued)

Table 2 Parameters for ionic Na^+ currents in patch clamp recording fitted by two state model

	Activation		Steady-state fast inactivation	
	k (mV/e-fold)	$V_{1/2}$ (mV)	*k* (mV/e-fold)	$V_{1/2}$ (mV)
WT ($n = 7$)	4.2 ± 0.4	−25.5 ± 1.4	4.6 ± 0.2	62.6 ± 0.8
R219K ($n = 8$)	5.0 ± 0.4	−22.5 ± 1.6	4.7 ± 0.3	63.7 ± 0.9

$V_{1/2}$ indicates the mid-point value of the steady-state fast inactivation curve (V-hinf) and the voltage dependence of activation (G-V). *k* is the slope factor. Errors indicate standard error of the mean.

right axis and Supplementary Fig. 3). Similarly, the inactivation gating behaviour of R219K channels was comparable to WT for steady-state voltage-dependence (Fig. 2A, left axis), as were the kinetics of entry to and recovery from inactivation (Fig. 2B). All gating parameters of Na^+ ionic currents are shown in Table 2.

Movement of the channel voltage-sensor in response to a change in membrane potential was measured as the charge-displacement current in the cut-open oocyte preparation (Fig. 2C–E), where the channel expression is higher and the clamp speed is faster. The voltage for the mid-point of charge displacement, as well as the steepness with voltage, were indistinguishable for WT and R219K channels, which is consistent with the expectations for a charge-conserving mutation of the voltage-sensor domain.

Gating pore current is small for $Na_V1.4$-R219K and undetectable for $Ca_V1.1$-R897K

The oocyte expression system was used to test for the presence of anomalous gating pore currents because the sensitivity is increased by the higher membrane expression of channel density compared to HEK293t cells. The Na-conducting pore was blocked by tetrodotoxin, and steady-state currents were measured for a series of test depolarizations from a holding value of −100 mV. The current amplitude was divided by the maximal charge-displacement, to normalize for the expression level of $Na_V1.4$ channels in each oocyte, and plotted as a function of test potential (Fig. 3A). An excess of inward current (negative) was detected at voltages of −80 mV and more negative, for oocytes expressing $Na_V1.4$-R219K channels compared to WT. This difference is consistent with a gating pore current. Moreover, the inward rectification of this current shows the gating pore current is activated by hyperpolarization, which is expected for a mutation in the outermost arginine of S4 (R219K). The inward displacement of the S4 segment with hyperpolarization brings the R219K mutation into the hydrophobic charge transfer centre, where a tight interaction with R219 normally prevents the leakage of ions. The amplitude of the R219K gating pore current, however, is much smaller than those observed in other HypoPP-2 mutant $Na_V1.4$ channels (Struyk and Cannon, 2007; Struyk *et al.*, 2008; Francis *et al.*, 2011).

The WT or R897K $Ca_V1.1$ channels were co-expressed with the $\alpha_2\delta$, β_1 and Stac3 subunits in oocytes. Membrane expression of WT channels was higher than for R897K mutant channels, based on the maximum charge displacement, 0.60 ± 0.074 nC ($n = 8$) and 0.29 ± 0.018 nC ($n = 8$), respectively. We are confident that the expression level of R897K channels was sufficient to detect a gating pore current because we had previously demonstrated robust gating pore currents for the HypoPP mutation $Ca_V1.1$-R528H when the maximum charge displacement was comparable (0.25 nC) (Wu *et al.*, 2018b). In an external Na^+ solution, the early current for oocytes expressing $Ca_V1.1$-R897K was not different from those expressing WT $Ca_V1.1$ (Fig. 3B). For many, but not all, anomalous gating pore conductances created by R/X missense mutations in S4, including $Ca_V1.1$-R528G (Wu *et al.*, 2018b), the gating pore current amplitude is dramatically enhanced in the presence of external guanidinium. We, therefore, tested $Ca_V1.1$-R897K in 60 mM external guanidinium, but again the steady-state current was identical to that observed in WT $Ca_V1.1$ (Fig. 3C). We conclude that under the expression conditions used in this study, there was no detectable gating pore current for $Ca_V1.1$-R897K channels.

Discussion

In this study, we report five cases of HypoPP from four different pedigrees in which a charge-preserving missense mutation (R/K) in S4 of a VSD was identified in $Ca_V1.1$

Figure 2 Continued
R219K mutation ($Na_V1.4$-R219K). The pulse protocol used is shown as inset in the left. (**D**, **E**) The relative charge moved by the voltage sensors (integral of charge-displacement current) is shown as a function of test potential in the Q–V relationship (**D**) and Tau–V relationship (**E**) for WT ($n = 5$) and $Na_V1.4$-R219K ($n = 5$), respectively. The time constant of gating currents were fitted by two-exponential fitting and plotted both fast components and slow ones separately (opened triangles and squares). The mean of time constant was calculated by the weighted average and are shown in filled marks.

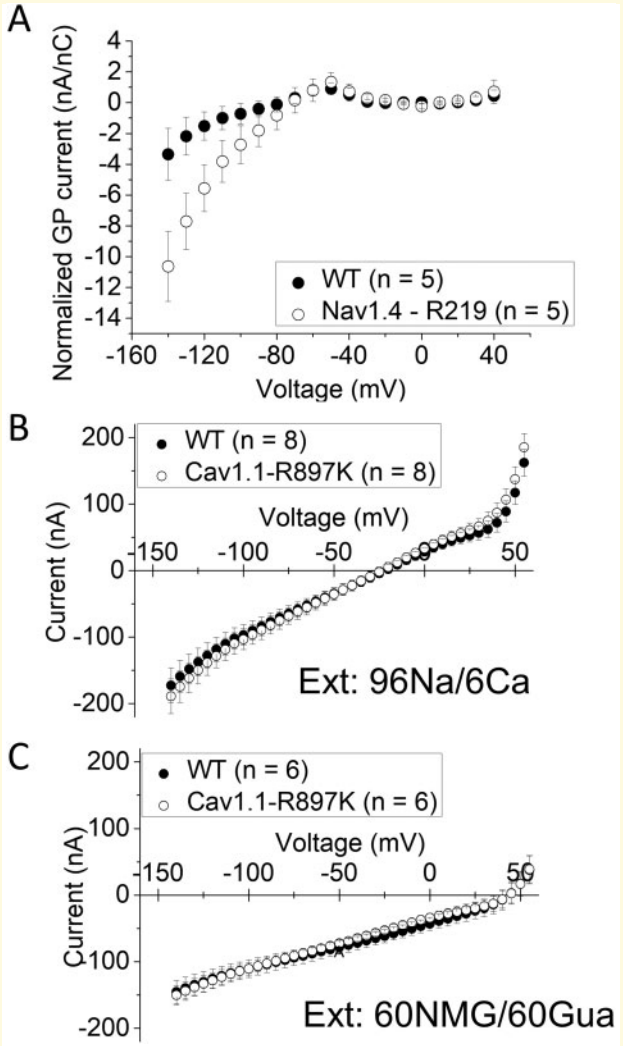


Figure 3 Gating pore current is small in $Na_V1.4$-R219K and undetectable in $Ca_V1.1$-R897K. (**A**) Gating pore current was observed for test potentials < -80 mV ($n = 5$, respectively). Leak currents recorded were subtracted by the linear fitted line from -20 mV to $+20$ mV in each oocyte. Error bars indicate standard error of the mean. No gating pore current was detected for oocytes expression $Ca_V1.1$-R897K in two different conditions: (**B**) external Na^+ or (**C**) a mixture of 60 mM *N*-methylglucamine and 60 mM guanidine. For (**B**), $n = 8$ oocytes, and for (**C**), $n = 6$ oocytes.

or $Na_V1.4$. Expression studies revealed a small gating pore current for $Na_V1.4$-R219K channels, but no detectable gating pore current for $Ca_V1.1$-R897K channels.

The clinical phenotypes of these R/K cases are typical for HypoPP

Recurrent episodic attacks of severe paresis with ictal hypokalaemia (<3.0 mmol/l) was observed in all five patients. Episodes were triggered by rest after exercise, ethanol or carbohydrate-rich meals, and clinical improvement occurred with acetazolamide, K supplementation or K-sparing diuretics. Missense variants were identified in all cases, with four having $Ca_V1.1$-R897K and one with $Na_V1.4$-R219K. These variants were not found in unaffected family members or in gene databases of subjects without neuromuscular disease. The familial transmission was demonstrated in one family with $Ca_V1.1$-R897K, and the others were *de novo*. Moreover, another missense mutation at this residue, $Ca_V1.1$-R897S, has been associated with a severe HypoPP phenotype (Chabrier *et al.*, 2008; Hanchard *et al.*, 2013). The prolonged CMAP exercise test was consistent with HypoPP [pattern V, with ≥40% decrement (Fournier *et al.*, 2004; Tan *et al.*, 2011)] for the $Na_V1.4$-R219K case and for one of the three $Ca_V1.1$-R897K patients who were tested, with the other two being borderline (25–31% decrement). Among the $Ca_V1.1$-R897K cases, 3 of 4 had clinical and imaging evidence of proximal myopathy, and in one a muscle biopsy showed vacuolar changes and T-tubular aggregates. The one case with $Na_V1.4$-R219K did not have fixed myopathy, but he is younger than the others at age 34. Taken together, the clinical data provide convincing evidence of typical HypoPP. The genetic variants segregate with affected individuals, and all occur at arginine residues in S4 segments, adjacent to established R/X HypoPP mutations. Therefore, we propose that the $Ca_V1.1$-R897K and $Na_V1.4$-R219K variants are disease-causing mutations.

The anomalous gating pore current typically found for HypoPP mutant channels was not detected in the R/K mutants

The gating pore current arising from non-conserved missense mutations at the first or second arginine in the S4 segments of $Ca_V1.1$ or $Na_V1.4$ has been a consistent finding in HypoPP mutant channels. Gating pore currents are present for all 11 HypoPP R/X mutations of $Na_V1.4$ reported to date (Sokolov *et al.*, 2007; Struyk and Cannon, 2007; Struyk *et al.*, 2008; Francis *et al.*, 2011; Bayless-Edwards *et al.*, 2018). Expression studies have been performed on 7 of the 9 HypoPP mutations identified in $Ca_V1.1$. Gating pore currents were observed for six of these mutant constructs: one in a knock-in mutant mouse (Wu *et al.*, 2012), four expressed in oocytes (Wu *et al.*, 2018*a*, *b*) which included the same mutant construct in the knock-in mouse, and another two by transient expression in mouse skeletal muscle (Fuster *et al.*, 2017*a*, *b*). This latter group includes the one atypical HypoPP mutation that is not located at an arginine in S4 (V876E). We did not detect a gating pore current for the HypoPP mutation $Ca_V1.1$-R900S expressed in oocytes, but another HypoPP mutation at this same R residue caused by substitution with a smaller amino acid, $Ca_V1.1$-R900G, did support a typical gating pore current

(Wu *et al.*, 2018*a*). In aggregate, these multiple studies provide compelling evidence that the gating pore current is an essential contributor to the pathogenesis of HypoPP.

A gating pore current was detected for $Na_V1.4$-R219K (Fig. 3A), but the amplitude was considerably small than those currents observed in other $Na_V1.4$ HypoPP mutant channels. For example, at the typical resting potential of skeletal muscle of −90 mV, the gating pore current for R219K was −2.8 nA/nC (Fig. 3A), whereas for our prior studies of HypoPP-2 mutations at R669, R672, or R1132, the gating pore current amplitude was −48.5 ± 13 nA/nC (range −17.3 to −99.7 nA/nC). A contributing factor to the small amplitude of the R219K gating pore current at −90 mV was the left-shifted (more negative) voltage for inward rectification which was about −80 mV. Similarly, we found a relatively negative rectification voltage (−45 mV) for the R669H channel, which consequently also had the smallest amplitude gating pore current (−17.3 nA/nC) amongst the HypoPP mutant channels in S4 of domain II. Both R219 and R669 are the outermost arginines in the S4 segments of domain I and II, respectively, and so a more negative membrane hyperpolarization is required to sufficiently shift the S4 segment far enough inward such that the mutant residue misaligns with the hydrophobic charge transfer centre to produce the leak. By comparison, the rectification voltage of the gating pore current for the R672 HypoPP mutant channels (second arginine from outside) is −25 mV (Struyk *et al.*, 2008).

We used our model simulation of fibre excitability (Cannon, 2018) to test whether the small-amplitude gating pore current observed for $Na_V1.4$-R219K is sufficient to cause the paradoxical depolarization that occurs in an attack of HypoPP. Figure 4 shows the membrane potential of a simulated fibre as the extracellular K is varied. In normal muscle for which there is no gating pore current (Fig. 4, solid line in black), the membrane potential becomes more negative (hyperpolarized) as K is reduced because of the Nernst potential for K shifts to more negative potentials (e.g. 4.5 mM to 3.5 mM). In extremely low $[K^+]$, 2.25 mM in this example, a paradoxical depolarization to −50 mV occurs because a reduction of the inward rectifier K^+ current is no longer able to maintain the normal resting potential. At this depolarized potential, the fibre is chronically refractory and inexcitable leading to flaccid paralysis. Such low values of $[K^+]$ do not normally occur in vivo, and so we do not have attacks of weakness. The addition of the gating pore current, however, shifts this catastrophic depolarization to higher $[K^+]$ that may overlap with the low physiologic range. The question is whether the small gating pore current observed for $Na_V1.4$-R219K is sufficient to cause susceptibility to HypoPP. As described above, the amplitude of the gating pore current at −90 mV for a typical $Na_V1.4$ HypoPP mutant channel expressed in a *Xenopus* oocyte is −48 nA/nC. This current density is equivalent to a

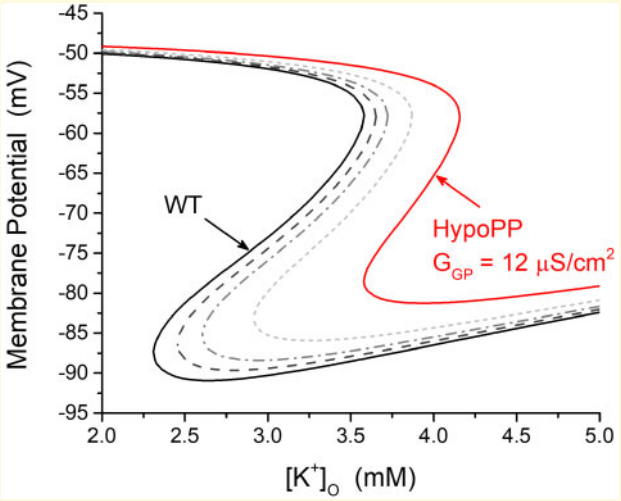


Figure 4 Simulation shows the small gating pore current for $Na_V1.4$-R219K is not predicted to cause susceptibility to HypoPP. Curves show membrane potential as the extracellular $[K^+]$ is varied, for a simulated muscle fibre. Black trace indicates the response for a normal fibre, without gating pore currents. In this case, paradoxical depolarization occurs only for extremely low $[K^+]_O$, < 2.25 mM. The red trace shows the response for a representative HypoPP fibre with a gating pore conductance, G_{GP} = 12 μS/cm², set at the mean value observed for S4 R/X HypoPP mutant channels of domains II and III. The gating pore current causes the right shift of the curve, such that paradoxical depolarization now occurs for $[K^+]_O$ in the low physiological range (e.g. 3.5 mM). The additional curves show the response of the membrane potential if the gating pore conductance is reduced to half (short-dashed line in light grey), one-fourth (dash-dot line in grey) or one-eighth (dashed line in dark grey) of the typical value for a HypoPP mutation (solid line in red). By comparison, the gating pore current for $Na_V1.4$-R219K at −90 mV is 17 times smaller than for other HypoPP mutant channels, which is insufficient to be a risk for HypoPP by the mechanism in this simulation.

slope conductance of 12 μS/cm² in a muscle fibre that is heterozygous for the HypoPP mutation, as in patients (Mi *et al.*, 2014). The addition of this gating pore leakage current now causes paradoxical depolarization in 3.5 mM $[K^+]$, which simulates the HypoPP phenotype (Fig. 4, solid line in red). If the amplitude of the gating pore current is reduced to 50% of the prior value, then depolarization is predicted to occur for $[K^+]$ = 2.9 mM, a low value that rarely occurs and so an attack of HypoPP is expected to be very infrequent. If the gating pore current is further reduced to one-fourth the typical value in HypoPP, then $[K^+]$ must be reduced to 2.6 mM for the fibre to depolarize. Similarly, with one-eighth the gating pore current, $[K^+]$ must be 2.4 mM for depolarization. The gating pore current for $Na_V1.4$-R219K at −90 mV was one-seventeenth the amplitude of a typical HypoPP mutant channel. According to our model simulations (Fig. 4), such a small leakage current is not predicted substantially shift the point of catastrophic

depolarization to a $[K^+]$ value different from normal muscle. Therefore, we conclude the small gating pore current observed for $Na_V1.4$-R219K is not sufficient to cause a risk for HypoPP.

We were unable to detect a gating pore current for $Ca_V1.1$-R897K in the oocyte expression system. This failure is not because of limited expression of the mutant channel in the membrane because the charge-displacement current (a functional measure of voltage-sensor activity in the membrane) was comparable to the value we achieved with $Ca_V1.1$-R528H HypoPP channels that had robust gating pore currents (Wu *et al.*., 2018b). Moreover, we used a physiological extracellular solution (primarily Na^+) and did not use $Ca_V1.1$ pore blockers (e.g. Co^{2+}) that might have inadvertently blocked a gating pore conductance. Chloride-free solutions were used to greatly reduce the contribution of endogenous Cl^- currents in oocytes, which interfere with the detection of a gating pore current. The charge-conserving R/K substitution raises the interesting theoretical possibility of a Cl^--selective gating pore leak. Although we did not test for such a possibility, a small leakage Cl^- current is not predicted to alter the excitability of a muscle fibre because the resting Cl^- conductance via ClC-1 channels is enormous. Other data clearly show that a missense mutation at $Ca_V1.1$-R897 is capable of supporting a gating pore current. The $Ca_V1.1$-R897S mutation has been identified in two *de novo* HypoPP cases, both of which had severe clinical phenotypes (Chabrier *et al.*, 2008; Hanchard *et al.*, 2013), and expression studies in oocytes revealed gating pore currents about 2× larger than values observed for the most common HypoPP mutation, $Ca_V1.1$-R528H (Wu *et al.*, 2018*a*). Another possibility is that the oocyte expression system is inadequate to reveal the creation of an anomalous gating pore conductance for $Ca_V1.1$-R897K channels. Many potential causes could be proposed: an interacting protein is missing, post-translational modification is different in the oocyte or an endogenous substance in the oocyte occludes the gating pore conductance. Expression studies in mammalian muscle fibres are needed to explore these possibilities.

Other hypotheses for pathological mechanisms of HypoPP

In addition to the anomalous gating pore current, other functional defects of HypoPP mutant channels have been reported. For HypoPP mutations of $Na_V1.4$ with typical clinical features, several loss-of-function defects have been reported. Inactivation (fast or slow) may be enhanced (Jurkat-Rott *et al.*, 2000; Struyk *et al.*, 2000), current density may be reduced, or the coupling of voltage-sensor movement to channel opening may be impaired (Mi *et al.*, 2014). While these loss-of-function changes would exacerbate a depolarization-induced inhibition of Na channel availability arising from the gating pore current, these loss-of-function defects alone do not cause depolarization-induced weakness in low potassium. Because the $Na_V1.4$-R219K variant was identified in a single proband and no genetic information is available for the parents, we cannot exclude the possibility R219K is a benign variant. If this were the case, then we propose the HypoPP phenotype may be from another gene (one-third of clinically definite HypoPP patients do not have an identified mutation in *CACNA1S* or *SCN4*) or in a non-coding region of *CACNA1S* or *SCN4A*. Atypical forms of HypoPP that are unusual because of coexisting myotonia or HyperPP have mixed gain-of-function and loss-of-function defects (Sugiura *et al.*, 2003; Kokunai *et al.*, 2018; Luo *et al.*, 2018). Alternative pathomechanisms are less apparent for HypoPP mutations of $Ca_V1.1$ without gating pore currents. The fundamental challenge is that $Ca_V1.1$ normally has little or no influence on the membrane resting potential, and so altered gating or expression level of mutant $Ca_V1.1$ is not expected to destabilize the resting potential. Two $Ca_V1.1$ mutations with convincing clinical phenotypes of typical HypoPP do not have detectable gating pore currents in the oocyte expression system: the charge-conserving CaV1.1-R897K herein and the non-conserved $Ca_V1.1$-R900S (Wu *et al.*, 2018*a*). These exceptions raise the possibility that another class of functional defect remains to be discovered for $Ca_V1.1$ HypoPP mutant channels.

Conclusion

This study describes five cases with typical clinical features of HypoPP, but with previously unreported charge-preserving amino acid substitutions at the outermost arginines in the voltage sensor of domain III in $Ca_V1.1$ or domain I in $Na_V1.4$. No gating pore current was detectable for $Ca_V1.1$-R897K, and only a small gating pore current was observed for $Na_V1.4$-R219K. Simulation studies suggest the small gating pore current for $Na_V1.4$-R219K is not sufficient to cause paradoxical depolarization in low K^+. These findings suggest additional functional defects, besides the gating pore leakage current, may cause susceptibility to HypoPP.

Supplementary material

Supplementary material is available at *Brain Communications* online.

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Competing interests

The authors report no competing interests.

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