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Enhanced inactivation and pH sensitivity of Na^+ channel mutations causing hypokalaemic periodic paralysis type II

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Summary

Hypokalaemic periodic paralysis (hypoPP) is a dominantly inherited muscle disorder characterized by episodes of flaccid weakness. Previous genetic studies revealed mutations in the voltage-gated calcium channel α1-subunit (CACNA1S gene) in families with hypoPP (type I). Electrophysiological studies on these mutants in different expression systems could not explain the pathophysiology of the disease. In addition, several mutations (Arg669His, Arg672His, Arg672Gly and Arg672Ser) in the voltage sensor of the skeletal muscle sodium channel α-subunit (SCN4A gene) have been found in families with hypoPP (type II). For Arg672Gly/His a fast inactivation defect was described, and for Arg669His an impairment of slow inactivation was reported. Except for the substitution for serine, we have now expressed all mutants in a human cell-line and studied them electrophysiologically. Patch–clamp recordings show an enhanced fast inactivation for all three mutations, whereas two of them reveal enhanced slow inactivation. This may reduce the number of functional sodium channels at resting membrane potential and contribute to the long-lasting periods of paralysis experienced by hypoPP patients. The gating of both histidine mutants (Arg669His, Arg672His) can be modulated by changes of extra- or intracellular pH. The inactivation defects of Arg669His and Arg672His can be alleviated by low pH to a significant degree, suggesting that the decrease of pH in muscle cells (e.g. during muscle work) might lead to an auto-compensation of functional defects. This may explain a delay or prevention of paralytic attacks in patients by slight physical activity. Moreover, the histidine residues may be the target for a potential therapeutic action by acetazolamide.

Keywords: channelopathies; paralysis; patch-clamp; S4 segment; voltage sensor

Abbreviations: AP = action potential; CACNA1S = skeletal muscle voltage-gated calcium channel α1-subunit; hypoPP = hypokalaemic periodic paralysis; hyperPP = hyperkalaemic periodic paralysis; SCN4A = skeletal muscle voltage-gated sodium channel α-subunit; WT = wild type

Introduction

Hypokalaemic periodic paralysis (hypoPP) is the most common form of periodic paralyses in man, although it is still a rare disease, with a prevalence of about 1 in 100 000. The major symptoms of hypoPP, i.e. episodes of generalized paralysis, are on average of longer duration than in a related disease, hyperkalaemic periodic paralysis (hyperPP); however, the differential diagnosis between the two syndromes is often difficult. Decisive for classification is the level of serum potassium during a paralytic attack, which may fall below 2 mmol/l in hypoPP. Clinical or electrical myotonia excludes the diagnosis of hypoPP (Lehmann-Horn *et al*., 1994).

Paralytic attacks occur after excessive intake of carbohydrates or several hours following strenuous exercise, commonly starting in the second half of the night, so that the awakening patient is unable to move, and symptom relief only occurs in the late morning. Slight physical activity can sometimes prevent or delay mild attacks. Independent of the occurrence of attacks, many patients develop a late onset,

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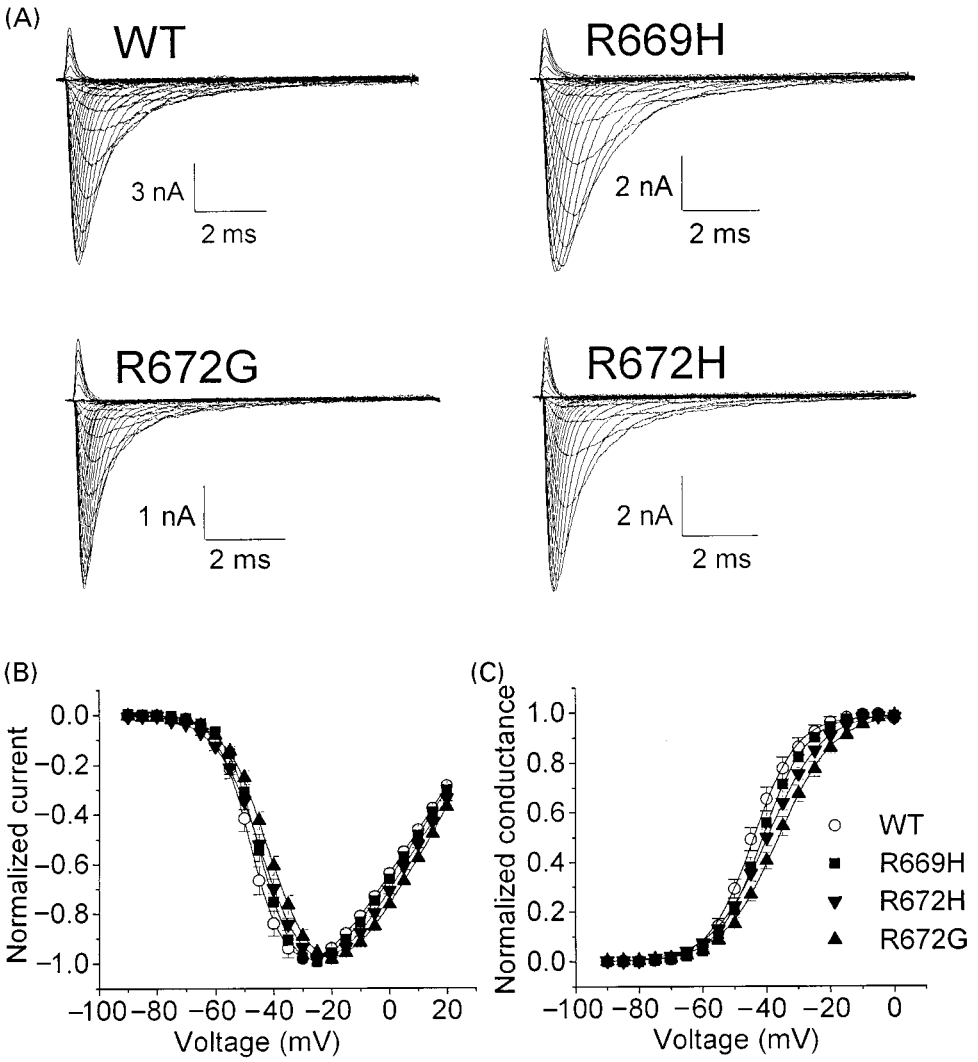

Fig. 1 (**A**) Sodium currents elicited by a family of 10 ms depolarizations from a –140 mV holding potential to voltages ranging from –90 to +65 mV in 5 mV steps were recorded from tsA201 cells expressing WT, Arg669His, Arg672His and Arg672Gly α_1-subunit channels. Corresponding current–voltage (**B**) (n = 10) and conductance–voltage (**C**) (n = 18) relationships.

progressive myopathy (Lehmann-Horn *et al*., 1994; Links *et al*., 1994).

Genetic studies of hypoPP revealed several point mutations in the gene encoding the L-type calcium channel (Jurkat-Rott *et al*., 1994; Ptacek *et al*., 1994). The L-type calcium channel functions as the voltage-sensor of the ryanodine receptor, which releases calcium from the sarcoplasmatic reticulum, initiating contraction (Melzer *et al*., 1995). Despite the knowledge of and functional studies on hypoPP-causing mutations, the pathophysiology of the disease remains unclear (Jurkat-Rott *et al*., 1998; Morrill *et al*., 1998; Morrill and Cannon, 1999).

Voltage-gated sodium channels are responsible for the initiation and propagation of action potentials in excitable cells. In order to regulate the action potential properly, the depolarizing sodium current needs to be quickly activated and inactivated. If inactivation is too slow or incomplete, the repolarizing phase of the action potential is delayed and a stable resting potential can not be maintained. This 'gain of function' mechanism is common for the sodium channel disorders of skeletal muscle like paramyotonia congenita, potassium-aggravated myotonia and hyperPP (Lehmann-Horn *et al*., 1991; Cannon *et al*., 1993; Lerche *et al*., 1993; Chahine *et al*., 1994; Mitrovic *et al*., 1994, 1995).

Recently, four mutations in the gene encoding the skeletal muscle sodium channel α-subunit (SCN4A gene) were reported. Our group linked five hypoPP families to SCN4A (Jurkat-Rott *et al*., 2000). Two mutations in that gene (Arg672Gly and Arg672His) were identified by functional expression studies as disease-causing mutations. For both mutations, an enhanced fast sodium channel inactivation was reported. Struyk *et al*. (2000) recently reported a defect of slow inactivation for the Arg669His mutation found previously by Bulman *et al*. (1999). The fourth mutation

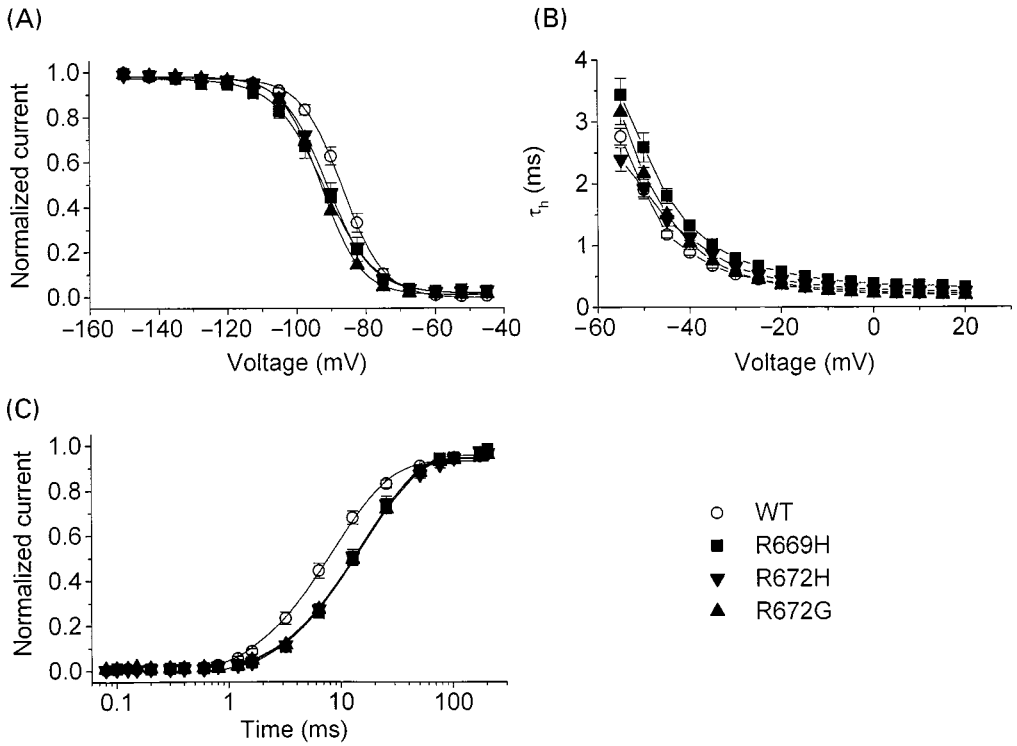

Fig. 2 (**A**) Steady-state fast inactivation was determined from a holding potential of –150 mV using a series of 300 ms prepulses from –150 to –45 mV in 7.5 mV increments prior to the test pulse of –20 mV. (**B**) Voltage dependence of time constants of fast inactivation time constants, τ_h, are given for all mutants (n = 9) and WT (n = 15). (**C**) Recovery from inactivation for a holding potential of –100 mV. Pulse protocol is given in the text.

(Arg672Ser) was also recently reported by two different groups (Davies *et al*., 2001; Sternberg *et al*., 2001), and has not yet been studied. All SCN4A gene mutations causing hypoPP neutralize a positively charged arginine in the voltage sensor S4 of domain II. Three of these mutations substitute the same residue, Arg672.

To prove whether sodium channel mutations associated with hypoPP families show a defect of fast or slow inactivation, or both, we here present a detailed study of the electrophysiological parameters of three SCN4A gene mutants: Arg669His, Arg672Gly and Arg672His. We also look at the effects of pH changes on the behaviour of the three mutations.

Material and methods

Mutagenesis and patch-clamp experiments

Site-directed mutagenesis was performed using an overlapping PCR (polymerase chain reaction)-based technique. Subsequently, the mutants were reassembled in the pRC/CMV plasmid (Invitrogen, San Diego, Calif., USA) for transfection by the calcium phosphate precipitation method in tsA201, a transformed human embryonic kidney cell line (HEK293). Cells were incubated at 37°C.

Standard whole-cell recording methods were used as described previously (Mitrovic *et al*., 1994, 1999). Individual cells were allowed to equilibrate for 10 min after achieving internal access before acquiring data. The pulse protocols are given in the Results and figure legends. Capacity transients were eliminated by a –P/4 protocol. Series resistance errors were <3 mV. Data were filtered at 3 kHz, and acquired using pCLAMP (Axon Instruments, Foster City, Calif., USA). Patch electrodes contained (in mM): 105 CsF, 35 NaCl, 10 EGTA, 10 Cs-HEPES, pH 7.4. The bath contained (in mM): 150 NaCl, 2 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 10 Cs-HEPES, pH 7.4. pH values were changed using CsOH. Corrections were made for liquid junction potentials. Most experiments were performed at room temperature (20–22°C). Whole-cell data were analysed by a combination of pCLAMP and ORIGIN (MicroCal) programs. Data are presented as means ± standard errors. Student's t-test was applied for statistical evaluation.

Results

We studied electrophysiologically three mutations recently reported in families with hypoPP. All three of them neutralize an outermost or second outermost positively charged arginine in the voltage sensor S4 in domain II of the SCN4A subunit by either a histidine (Arg669His and Arg672His) or a glycine (Arg672Gly). Figure 1 shows typical recordings of sodium currents after a series of depolarizing pulses between –90 and

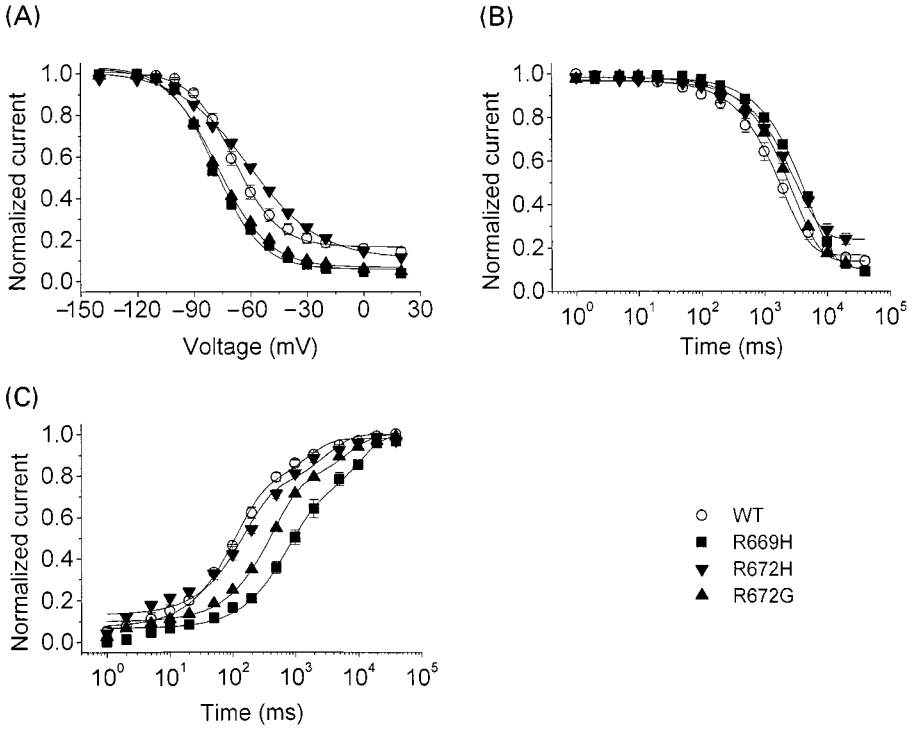

Fig. 3 (**A**) Cumulative steady-state slow inactivation was determined from a holding potential of –140 mV using a series of 30 s prepulses between –140 and +20 mV prior to the test pulse of –20 mV. (**B**) Entry into slow inactivated state for all four clones. (**C**) Recovery from slow inactivation for a holding potential of –140 mV was determined by a 30 s depolarization to –20 mV followed by a variable duration return to –140 mV.

+65 mV from a holding potential of –140 mV. Currents through mutant channels had similar kinetics when compared with wild type (WT). The corresponding I–V and G–V curves are shown in Fig. 1B and C. Mutant channels show a right shift and a reduction in slope of the G–V curves, as expected for a neutralization of one positive charge in the voltage sensor S4. For the Arg672His, Arg672Gly and Arg669His mutations this shift was 4, 8 and 3 mV, respectively. The difference was significant ($P < 0.03$) for Arg672His and Arg672Gly, but not for Arg669His.

All mutants stabilize fast inactivation

Steady-state fast inactivation was tested by the following protocol: a series of 300 ms depolarizing prepulses from –150 to –45 mV in 7.5 mV steps was used in order to inactivate the channels prior to the test pulse to –20 mV. Figure 2A shows the steady-state inactivation curve for WT and mutant channels. Fits to a Boltzmann function revealed a significant left shift for all mutant channels. For Arg669His, this shift was 5 mV [$V_{0.5}$ (in mV): 92 ± 2 (Arg669His) ($n = 9$) versus 87 ± 1 (WT) ($n = 16$), $P < 0.05$], the slope of the curve was slightly decreased [k: 6.3 ± 0.3 (Arg669His) ($n = 9$) versus 5.3 ± 0.2 (WT) ($n = 16$), $P < 0.01$]. For Arg672His and Arg672Gly, the steady-state inactivation curve was also shifted to the left by ~5 mV [$V_{0.5}$ (in mV): 93 ± 1, $P < 0.0001$, $n = 22$ (Arg672Gly), and 91 ± 1, $P < 0.05$, $n = 17$ (Arg672His)]. These shifts were somewhat smaller than initially reported (Jurkat-Rott *et al*., 2000); however, they were still significantly different from the WT. The left-shift of steady-state inactivation enhances inactivation and may contribute to paralysis in patients with hypoPP by reducing the number of available sodium channels and the excitability of the muscle fibre membrane. The inactivation time constants were not significantly different between the various clones (Fig. 2B).

Consistent with the left-shift of steady-state fast inactivation, recovery from fast inactivation studied at –100 and –120 mV was significantly slower for the mutant channels. The following pulse protocol was used: a 100 ms pulse to –20 mV was used to inactivate sodium channels. Recovery was measured at mentioned potentials for increasing durations prior to the test pulse to –20 mV. At –100 mV the rate of recovery was approximately twofold slower for the Arg669His compared with the WT [τ_{rec} at –100 mV (in ms): 16.7 ± 1.5 (Arg669His) ($n = 6$) versus 9.7 ± 0.9 (WT) ($n = 9$), $P < 0.01$] (Fig. 2C). For Arg672Gly and Arg672His, recovery from fast inactivation was also slower, e.g. at –100 mV τ_{rec} was 16.9 ± 1.3 and 16.6 ± 1.9 ms, respectively.

Arg669His and Arg672Gly stabilize slow inactivation

Steady-state slow inactivation was studied using the following protocol: channels were inactivated by a 30 s pulse to the

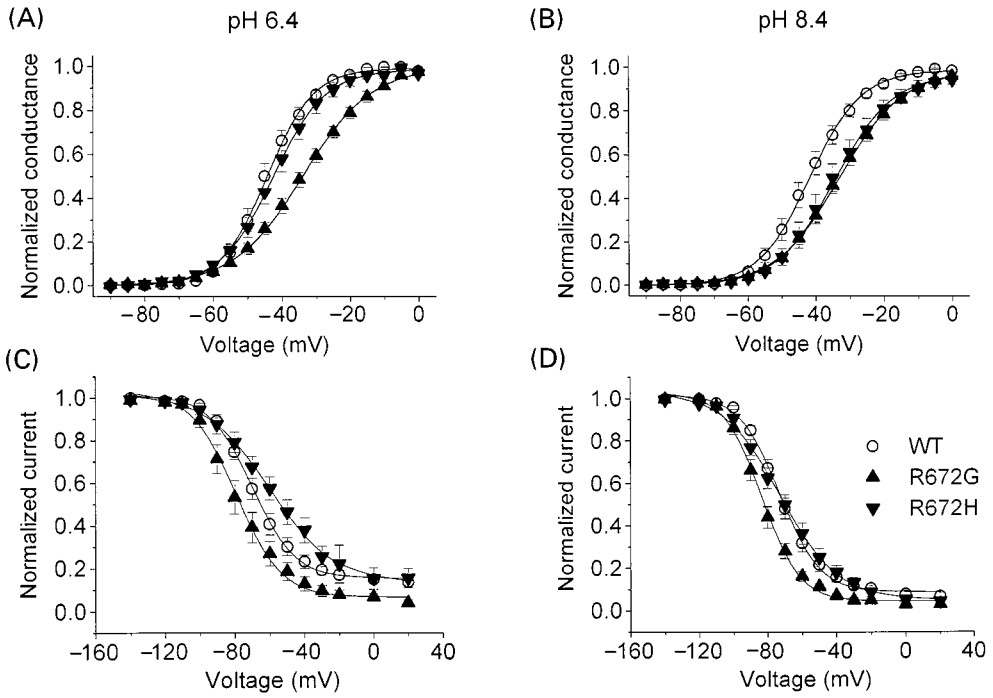

Fig. 4 Effects of intracellular pH on Arg672Gly and Arg672His mutations. Shown are steady-state activation (**A**, **B**) and slow inactivation (**C**, **D**) at pH 6.4 and 8.4, respectively.

potentials listed in Fig. 3A. Using this pulse ~95% of sodium channels entered the slow inactivated state. We let the channels recover from fast inactivation for 20 ms at –140 mV prior to the test pulse. Between two episodes channels were not recovered from slow inactivation. As shown in Fig. 3A, the quasi-steady-state slow inactivation curve for the Arg669His channels was shifted to the left by 11 mV [$V_{0.5}$ (in mV): –79 ± 1 (Arg669His) (n = 8) versus –68 ± 2 (WT) (n = 11), $P < 0.001$]. This shift could be due to either a faster entry into or a slower recovery from the slow inactivated state. Whereas the entry was not significantly different between WT and mutant channels (Fig. 3B), recovery from slow inactivation was 4-fold slower [$\tau_{recslow2}$ (in s): 5.2 ± 1.1 (Arg669His) (n = 3) versus 1.3 ± 0.2 (WT) (n = 6), $P < 0.05$]. Thus, the Arg669His mutation not only enhances fast inactivation, but also stabilizes slow inactivation. In hypoPP muscle, this defect should further diminish the number of available sodium channels, not allowing them contribute to the action potential.

For the Arg672Gly mutant, steady-state slow inactivation was shifted to the left by 10 mV [$V_{0.5}$ (in mV): –78 ± 2 (Arg672Gly) (n = 4) versus –68 ± 2 (WT) (n = 11), $P < 0.01$]. For Arg672His mutant, the slow inactivation curve showed a large decrease in slope [k: 19.2 ± 1.2 (Arg672His) versus 11.3 ± 0.4 (WT)] but no left-shift (Fig. 3A).

Figure 3 shows entry into (Fig. 3B) and recovery from (Fig. 3C) the slow inactivated state. As expected, recovery from slow inactivation was slower ($\tau_{recslow2}$ 4.1 ± 0.7 s) for the Arg672Gly compared with WT. For Arg672His, recovery was similar to WT channels; the entry into slow inactivated state was shifted slightly to the right.

Current density

Since the expression level of mutant channels in the muscle membrane may also determine the severity of the clinical phenotype (Mitrovic *et al*., 1999) we studied the density of mutant channels in our expression system. For the Arg669His mutant the mean current amplitude normalized to the cell capacitance was approximately twofold reduced compared with WT [368 ± 72 (n = 9) versus 690 ± 101 (n = 13) pA/pF, $P < 0.05$]. For the Arg672His mutant (n = 18), the current density was 222 ± 40 pA/pF, and for Arg672Gly (n = 10), 263 ± 28 pA/pF ($P < 0.001$) when compared with the WT. The culturing at pH 6.9 showed a similar reduction of current density for both WT and the two histidine mutants [in pA/pF: WT 88 ± 16 (n = 11), Arg669His 39 ± 5 (n = 7), Arg672His 35 ± 3 (n = 6), $P < 0.05$].

Effects of pH on channel gating

As shown above, the substitution of the outermost arginine by a histidine in the S4 segment of domain II of SCN4A enhances both fast and slow inactivation and may explain the pathophysiology of weakness in patients with hypoPP. Most of the patients with hypoPP reported in the literature show

benefit from the use of acetazolamide (Griggs *et al*., 1970), as also has been confirmed for some of our patients carrying sodium channel mutations (our unpublished data). However, the mechanism of action of acetazolamide is not known. A possible mechanism could be the regulation of potassium ion and proton concentration. In order to determine whether a change of the proton concentration has an influence on the gating of SCN4A mutants, we used pH values ranging from 6.4 to 8.4 in bath or pipette solution. The change of extracellular pH has profound effects on steady-state activation and inactivation of the WT channels, probably due to the change of surface potential but also to a direct block of sodium channels (Woodhull, 1973). The effect of intracellular pH on WT sodium channels is rather small.

Two of the mutations that we studied substitute a histidine for a positively charged arginine. A histidine residue may accept and release protons depending on the pH of the environment. At high pH histidine is mostly neutral, at low pH it may be protonated and become positive like the WT arginine. Therefore, we studied the effects of pH on mutant channels. According to experiments about exposure of the outermost and second outermost arginines in domain IV (Yang *et al*., 1996), we studied Arg699His upon changes of extracellular pH, and Arg672Gly and Arg672His upon changes of intracellular pH. First, we observed the effects of pH on activation. The G–V curve for the neutral Arg672Gly was shifted to the right by 8 mV compared with WT, and was not significantly influenced with variation of intracellular pH. This is in accordance with a neutralization of one positive charge in the voltage sensor S4 in domain II (D2/S4). At a pH of 7.4, histidine residues are partly charged and the activation curve for Arg672His was located between WT and Arg672Gly. A decrease of intracellular pH to 6.4 caused a left-shift of the Arg672His G–V curve, which was at this point almost identical to that of the WT. At this pH a larger number of the histidines should be protonated compared with at pH 7.4 (p*K* for histidine is 6.5). At a very high pH (8.4), where histidine is deprotonated, the Arg672His G–V behaved like a neutral glycine mutant (Fig. 4A). Altogether, the right-shift of the G–V curve as observed for the mutant channels can be explained by a neutralization of the positively charged arginine by glycine and histidine.

Steady-state fast inactivation was not modified by changes of intracellular pH. The increase of intracellular pH from 6.4 to 8.4 leads to a 12 mV left-shift of steady-state slow inactivation for Arg672His (Fig. 4B) further stabilizing the slow inactivated state. The steepness of the curve did not change with pH, suggesting that the large histidine residue disturbs the movement of D2/S4 not only by reducing the charge, but also through allosteric effects.

The extracellular pH modification of the Arg669His revealed similar results. At high pH the WT channels showed a 6 mV left shift of the steady-state activation curve (data not shown). The most simple explanation of this behaviour is that WT channels open more easily when the surface charge is reduced (pH 8.4). At the same pH, the Arg669His mutant shows a 12 mV right-shift of the G–V curve, suggesting that a deprotonated histidine reduces the probability of D2/S4 moving outward. At low pH, the mutant channels show a left-shift of the G–V curve. This shift is even larger than for the WT channels, suggesting that not only the charge modulation but also the conformational change of the D2/S4 is responsible for this effect. The increase of extracellular pH caused a significant 11 mV left-shift of the steady-state slow inactivation [$V_{0.5}$ for Arg669His (in mV): –88 ± 3 (pH 8.4) (n = 5) versus –77 ± 1 (pH 6.4) (n = 6), $P < 0.01$].

The pH experiments show that the Arg669His residue appears on the extracellular side of the membrane and can be modified by protons (this study), as well as with *N*-biotinylaminoethyl methanethiasulphonate reagents (Mitrovic *et al*., 1998). The Arg672His mutant can be modified by intracellular protons, suggesting that it appears on the internal side of the membrane. Thus it seems that the D2/S4 moves in a small crevice similar to the D4/S4 (Yang *et al*., 1996).

Effects of potassium on gating of R669H

HypoPP can be differentiated from hyperPP by measuring serum potassium during attacks. Furthermore, muscle weakness *in vitro* may be triggered in hypoPP by lowering potassium concentration. In order to determine whether there is a direct effect of low potassium on sodium channel mutants two potassium concentrations, 2 and 5 mM were used in extracellular solution. No significant difference in gating of WT or mutant channels was observed (data not shown), suggesting that changes of extracellular potassium trigger attacks in hypoPP and hyperPP in a different way (see Discussion).

Discussion

Recently, five families with hypoPP were linked to the gene encoding SCN4A, and two point mutations, Arg672Gly and Arg672His, were identified (Jurkat-Rott *et al*., 2000). Bulman *et al*. (1999) reported a Arg669His mutation in one hypoPP family. All of the mutations replace a positively charged arginine by a histidine or a glycine in the voltage sensor S4 of domain II (D2/S4).

The main electrophysiological finding for all three sodium channel mutants is a 5 mV left-shift of the steady-state fast inactivation curve. The consequence is enhanced fast inactivation, causing a reduction in the number of functional sodium channels in the cell membrane. The largest defect is found at normal resting membrane potentials, e.g. at –85 mV the number of available sodium channels drops from ~60% for the WT to 35% for the mutant. For the Arg669His and Arg672Gly mutations, an additional 10 mV left-shift of the steady-state slow inactivation curve was found. Enhanced slow inactivation may lead to a further reduction of functional sodium channels in the membrane. Since the excitability of muscle fibres depends on the availability of sodium channels and inactivated sodium channels are not able to contribute to

the initiation and the propagation of action potentials, the hypoPP muscle fibres may become hypo- or inexcitable.

The Arg669His mutant was recently studied by Struyk *et al*. (2000) and the authors reported only a defect of slow inactivation. The left-shift of steady-state slow inactivation was similar to the shift that we observed. In contrast to our study, the left-shift of the fast inactivation curve was not significant, although it was present (–3 mV). Since the other two hypoPP mutations show a similar shift of the steady-state inactivation, we believe that it is important in the pathophysiology of hypoPP.

An additional pathogenetically important finding in biopsied hypoPP muscle fibres is a reduced density of sodium channels. Namely, the action potential (AP) recordings showed a markedly reduced maximal amplitude of APs in the hypoPP muscle compared with the control (Jurkat-Rott *et al*., 2000). If the amplitude decrease is only due to the reduction of the functional channels, which are inactivated at resting membrane potential, one should expect a restoration of the AP amplitude with more negative holding potentials where sodium channels recover from inactivation more easily. Surprisingly there was only a small increase in the maximal amplitude of APs at hyperpolarized membrane (e.g. –95 mV), suggesting that the density of sodium channels in hypoPP muscle also has to be reduced. In accordance with this finding, an approximate two to fourfold decrease of the sodium current amplitude for the hypoPP mutants was found in tsA201 cells when normalized to the cell capacitance (Jurkat-Rott *et al*., 2000; this study). The reduction of pH to 6.9 resulted in a similar reduction of current density for both WT and the two histidine mutants. This suggests that metabolic acidosis as obtained by acetazolamide probably exerts its therapeutic effect only by changing the steady-state properties of the histidine mutants, and not their expression level.

An impaired expression of the mutant gene or reduced open probability have been proposed to explain the reduction of current density. Although this defect may explain a deformation of APs as observed in hypoPP muscle, the expression of sodium channels in a cell line may not parallel the expression in native muscle. On the other hand, a lower expression of mutant channels was previously shown in native muscle specimens of patients with the related disease paramyotonia congenita. We proposed that the clinical phenotype may vary with a variable expression of the mutant protein (Mitrovic *et al*., 1999).

A characteristic feature for most of the hyperPP-causing mutants is a so-called persistent current, an aberrant sodium inward current caused by an inactivation defect (Lehmann-Horn *et al*., 1987, 1991; Cannon *et al*., 1991, 1993). The consequence of the inadequate closing of mutant sodium channels is an additional depolarization of the cell membrane. Depending on the extent of depolarization, both symptoms of hyper- and hypoexcitability may be induced. A slight membrane depolarization would cause a hyperexcitability with a clinical phenotype of myotonia and large sustained depolarization would induce muscle weakness. In contrast to this 'gain of function' mechanism, hypoPP mutations show a 'loss of function', since they reduce the number of excitable sodium channels by enhanced inactivation and reduced expression.

Although our results can explain the susceptibility of the hypoPP muscle for weakness the mystery of the hypoPP is only partly resolved. For example, we do not know how mutations in the same sodium channel gene lead both to hyperPP and to hypoPP. We can speculate on the hyperkalaemic phenotype in hyperPP as a consequence of a persistent sodium current, which shifts potassium ions from intracellular towards extracellular space. In contrast, hypokalaemia in hypoPP results from the physiological effect of glucose intake and the release of insulin, which stimulates the sodium–potassium pump and shifts potassium ions from the extracellular space into the intracellular compartment. In contrast to the membrane hyperpolarization observed in normal muscle, the hypokalaemia causes sustained depolarization of hypoPP fibres and initiates the attack (Rüdel *et al*., 1984). It was recently proposed that this hypokalaemia-induced depolarization is caused by reduction of an outward potassium current in 'low-potassium'. The role of SCN4A gene mutations in that process is not clear. In any case the sustained depolarization would induce inactivation of both mutant and WT SCN4A subunits and render the cell inexcitable, i.e. the patient paralysed.

It is very interesting that all SCN4A mutations causing hypoPP are localized in the voltage sensor S4 of domain II. Very recently, two groups reported the third substitution at position Arg672. In two hypoPP families, an Arg672Ser mutation was found. It is not clear why this particular residue and the S4 segment in domain II are so important for the pathogenesis of hypoPP. Experiments using the cysteine scanning methodology introduced by Falke *et al*. (1988) and Akabas *et al*. (1992) will probably give further insight into the functional significance of the D2/S4 mutation. In studies of S4 function, a cysteine is substituted for a selected S4 residue by site-directed mutagenesis, and the expressed channel is then exposed to a hydrophilic cysteine reagent while monitoring its biophysical properties (Yang and Horn, 1995; Yang *et al*., 1996; Mitrovic *et al*., 1998). The consequence of the reaction between the introduced cysteine residue and the reagent is a change in either activation or inactivation of the channel. It has been proposed that the function of S4 segments is specialized. Both S4 segments in domains I and II should play an important role in activation (Chen *et al*., 1996; Mitrovic *et al*., 1998; Cha *et al*., 1999), whereas the S4 segments in domains III and IV would be important for both activation and inactivation. Our study emphasizes the importance of D2/S4 not only in activation, but also in fast inactivation. In addition, similar to D4/S4 (Mitrovic *et al*., 2000), D2/S4 seems to play an important role in slow inactivation.

Our data show that the behaviour of histidine mutants may be modified by pH. The increase of pH from 6.4 to 8.4 leads to a 12 mV left-shift of steady-state slow

inactivation for Arg672His and a 10 mV left-shift for Arg669His, suggesting that the pH increase may induce or aggravate the defect of slow inactivation, indirectly reducing the excitability of the muscle fibre and triggering the paralytic attack. In contrast, a pH decrease in the muscle cell would lead to alleviation of the paralytic attack. For example, a reduction of pH in the muscle by slight physical activity could even prevent the attack. This is exactly what many hypoPP patients report.

Concerning these findings and assuming that acetazolamide mechanism of action is connected with pH regulation, we would predict that patients carrying a Arg672Gly mutation, which cannot be modulated by pH, would not benefit from acetazolamide. Sternberg and colleagues reported a very severe hypoPP phenotype in one family carrying the Arg672Gly sodium channel mutation (Sternberg *et al.*, 2001). The frequency and severity of attacks even increased with acetazolamide use. In contrast, patients with the Arg672His mutation show an alleviation of symptoms when acetazolamide is used (unpublished data).

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References

Akabas MH, Stauffer DA, Xu M, Karlin A. Acetylcholine receptor channel structure probed in cysteine-substitution mutants. Science 1992; 258: 307–10.

Bulman DE, Scoggan KA, van Oene MD, Nicolle MW, Hahn AF, Tollar LL, et al. A novel sodium channel mutation in a family with hypokalemic periodic paralysis. Neurology 1999; 53: 1932–6.

Cannon SC, Brown RH, Corey DP. A sodium channel defect in hyperkalemic periodic paralysis: potassium-induced failure of inactivation. Neuron 1991; 6: 619–26.

Cannon SC, Brown RH, Corey DP. Theoretical reconstruction of myotonia and paralysis caused by incomplete inactivation of sodium channels. Biophys J 1993; 65: 270–88.

Cha A, Ruben PC, George AL Jr, Fujimoto E, Bezanilla F. Voltage sensors in domains III and IV, but not I and II, are immobilized by Na+ channel fast inactivation. Neuron 1999; 22: 73–87.

Chahine M, George AL, Zhou M, Ji S, Sun W, Barchi RL, et al. Sodium channel mutations in paramyotonia congenita uncouple inactivation from activation. Neuron 1994; 12: 281–94.

Chen LQ, Santarelli V, Horn R, Kallen RG. A unique role for the S4 segment of domain 4 in the inactivation of sodium channels. J Gen Physiol 1996; 108: 549–56.

Davies N, Eunson L, Samuels M, Wise R, Hanna M. A molecular genetic study of hypokalemic periodic paralysis in the UK – a new skeletal muscle sodium channel gene (SCN4A) mutation [abstract]. J Neurol 2001; 248 Suppl 2: II/57–II/58.

Falke JJ, Dernburg AF, Sternberg DA, Zalkin N, Milligan DL, Koshland DE. Structure of a bacterial sensory receptor. A site-directed sulfhydryl study. J Biol Chem 1988; 263: 14850–8.

Griggs RC, Engel WK, Resnick JS. Acetazolamide treatment of hypokalemic periodic paralysis. Prevention of attacks and improvement of persistent weakness. Ann Intern Med 1970; 73: 39–48.

Jurkat-Rott K, Lehmann-Horn F, Elbaz A, Heine R, Gregg RG, Hogan K, et al. A calcium channel mutation causing hypokalemic periodic paralysis. Hum Mol Genet 1994; 3: 1415–19.

Jurkat-Rott K, Uetz U, Pika-Hartlaub U, Powell J, Fontaine B, Melzer W, et al. Calcium currents and transients of native and heterologously expressed mutant skeletal muscle DHP receptor alpha1 subunits (R528H). FEBS Lett 1998; 423: 198–204.

Jurkat-Rott K, Mitrovic N, Hang C, Kouzmekine A, Iaizzo P, Herzog J, et al. Voltage-sensor sodium channel mutations cause hypokalemic periodic paralysis type 2 by enhanced inactivation and reduced current. Proc Natl Acad Sci USA 2000; 97: 9549–54.

Lehmann-Horn F, Rüdel R, Ricker R. Membrane defects in paramyotonia congenita (Eulenburg). Muscle Nerve 1987; 10: 633–41.

Lehmann-Horn F, Iaizzo PA, Hatt H, Franke C. Altered gating and conductance of Na^+ channels in hyperkalemic periodic paralysis. Pflügers Arch 1991; 418: 297–9.

Lehmann-Horn F, Engel AG, Ricker K, Rüdel R. The periodic paralyses and paramyotonia congenita. In: Engel AG, Franzini-Armstrong C, editors. Myology. 2nd ed. New York: McGraw-Hill; 1994. p. 1304–34.

Lerche H, Heine R, Pika U, George AL, Mitrovic N, Browatzki M, et al. Human sodium channel myotonia: slowed channel inactivation due to substitutions for a glycine within the III–IV linker. J Physiol (Lond) 1993; 470: 13–22.

Links TP, Smit AJ, Molenaar WM, Zwarts MJ, Oosterhuis HJ. Familial hypokalemic periodic paralysis. Clinical, diagnostic and therapeutic aspects. J Neurol Sci 1994; 122: 33–43.

Melzer W, Herrmann-Frank A, Luttgau HC. The role of Ca^{2+} ions in excitation-contraction coupling of skeletal muscle fibres. [Review]. Biochim Biophys Acta 1995; 1241: 59–116.

Mitrovic N, George AL, Heine R, Wagner S, Pika U, Hartlaub U, et al. K^+-aggravated myotonia: destabilization of the inactivated state of the human muscle Na^+ channel by the V1589M mutation. J Physiol 1994; 478: 395–402.

Mitrovic N, George AL, Lerche H, Wagner S, Fahlke C, Lehmann-Horn F. Different effects on gating of three myotonia-causing mutations in the inactivation gate of the human muscle sodium channel. J Physiol (Lond) 1995; 487: 107–14.

Mitrovic N, George AL Jr, Horn R. Independent versus coupled inactivation in sodium channels. Role of the domain 2 S4 segment. J Gen Physiol 1998; 111: 451–62.

Mitrovic N, George AL Jr, Rüdel R, Lehmann-Horn F, Lerche H. Mutant channels contribute <50% to Na^+ current in paramyotonia congenita muscle. Brain 1999; 122: 1085–92.

Mitrovic N, George AL Jr, Horn R. Role of domain 4 in sodium channel slow inactivation. J Gen Physiol 2000; 115: 707–18.

Morrill JA, Cannon SC. Effects of mutations causing hypokalaemic periodic paralysis on the skeletal muscle L-type Ca^{2+} channel expressed in Xenopus laevis oocytes. J Physiol (Lond) 1999; 520: 321–36.

Morrill JA, Brown RH Jr, Cannon SC. Gating of the L-type Ca channel in human skeletal myotubes: an activation defect caused by the hypokalemic periodic paralysis mutation R528H. J Neurosci 1998; 18: 10320–34.

Ptacek LJ, Tawil R, Griggs RC, Engel AG, Layzer RB, Kwiecinski H, et al. Dihydropyridine receptor mutations cause hypokalemic periodic paralysis. Cell 1994; 77: 863–8.

Rüdel R, Lehmann-Horn F, Ricker K, Küther G. Hypokalemic periodic paralysis: in vitro investigation of muscle fiber membrane parameters. Muscle Nerve 1984; 7: 110–20.

Sternberg D, Maisonobe T, Jurkat-Rott K, Nicole S, Launay E, Chauveau D, et al. Hypokalaemic periodic paralysis type 2 caused by mutations at codon 672 in the muscle sodium channel gene SCN4A. Brain 2001; 124: 1091–9.

Struyk AF, Scoggan KA, Bulman DE, Cannon SC. The human skeletal muscle Na channel mutation R669H associated with hypokalemic periodic paralysis enhances slow inactivation. J Neurosci 2000; 20: 8610–17.

Woodhull AM. Ionic blockage of sodium channels in nerve. J Gen Physiol 1973; 61: 687–708.

Yang N, Horn R. Evidence for voltage-dependent S4 movement in sodium channels. Neuron 1995; 15: 213–18.

Yang N, George AL, Horn R. Molecular basis of charge movement in voltage-gated sodium channels. Neuron 1996; 16: 113–22.

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