

# **In vivo Sodium Channel Structure/Function Studies: Consecutive Arg1448 Changes to Cys, His, and Pro at the Extracellular Surface of IVS4**

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Structure/function relationships in ion channels have been intensively studied through expression of cloned channel subunits in heterologous cellular environments. Considerable information has been gleaned via this approach. However, it is difficult to know if the residues and regions identified as important retain this prominent role in vivo: there are many differences between heterologous systems and functioning nerves and muscle in vivo, any one of which is likely to affect channel function. Examples of such variables include glycosylation status of the channel protein, association of muscle-specific membrane or cytoskeletal proteins, and fluctuations of intracellular and extracellular fluid milieu as a function of fluctuating cellular physiology. The identification of single amino acid changes in the voltage-sensitive muscle sodium channel  $\alpha$  subunit in human and horse genetic disease has permitted a new approach to the study of structure/function relationships in ion channels. Importantly, the interactions between the environment and the abnormal channel can be studied in this in vivo system. Here we report the identification of a novel human sodium channel mutation (R1448P), which causes a severe type of cold-sensitive myotonia and weakness. This patient is compared to a series of other patients having R1448C, and R1448H mutations. We show that the severity of the amino acid change correlates with the severity of clinical symptoms. This data shows that different amino acid replacements in the extracellular surface of domain IV S4 are important for channel function, despite the paucity of heterologous expression data suggesting functional importance of this region. The extreme cold sensitivity of the proline substitution at R1443 suggests that cold temperatures may affect the structural integrity of the channel, and that proline may destabilize the normal structure.

## **Introduction**

A group of inherited neurological disorders share a propensity for involuntary repetitive firing of muscle (myotonia), with or without associated episodic weakness

(periodic paralysis). Nondystrophic myotonias generally do not show progressive muscle destruction as a clinical feature. Nearly all of the inherited primary types of nondystrophic myotonia are transmitted within families as a dominant trait; patients are heterozygous, with both a normal and abnormal gene. Most dominantly inherited mutations cause a "change-of-function" of the corresponding protein product; the mutant gene can still produce a protein product, however the product does not function correctly. The abnormal protein initiates a cascade of events that leads to the clinical phenotype.

The nondystrophic myotonias have been divided into subgroups based on clinical and laboratory features (Rude1, 1986). Hyperkalemic periodic paralysis (HyperPP) shows attacks of weakness or paralysis provoked by rest after exercise or ingestion of potassium salt, with most patients showing evidence of interictal myotonia. First described in humans in the 1950's (Gamstorp, 1956), HyperPP is also prevalent in Quarter horses with ~1/200 (0.5%) of the 3 million registered horses having the trait (Spier, Carlson, Holliday, Cardinet, and Pickar, 1990). Paramyotonia congenita (PC) shows attacks of myotonia induced by low temperatures, associated with or followed by some weakness. Thomsen's myotonia shows myotonia most often accompanied by stiffness, without weakness or paralysis. Becker's myotonia is clinically similar to Thomsen's although it is more frequently associated with transient weakness. It is the only nondystrophic myotonia inherited as a recessive trait. Hypokalemic periodic paralysis does not show myotonia, shows reduced penetrance in women, and has attacks induced by carbohydrate or sodium intake, and insulin or epinephrine injection. Patients frequently show a progressive myopathy, and attacks decrease in severity and frequency with age.

Recently, the clinical subtypes of nondystrophic myotonias have resolved into three types of genetic disorders involving sodium, chloride, and calcium channels. Different change-of-function mutations of the voltage-sensitive sodium channel cause most cases of PC and HyperPP (both human and horse) (Rojas, Wang, Schwartz, Hoffman, Powell, and Brown, 1991; Ptacek, George, and Griggs, 1991; McClatchey, Van den Berg, Pericak-Vance, Raskind, Verellen, McKenna-Yasek, Rao, Haines, Bird, Brown, and Gusella, 1992; Rudolph, Spier, Byrn, Rojas, Bernoco, and Hoffman, 1992), loss-of-function mutations of the voltage-sensitive muscle chloride channel appear to cause many cases of Thomsen's and Becker's myotonia (Koch, Steinmeyer, Lorenz, Richer, Wolf, Otto, Zoll, Lehmann-Horn, Grzeschik, and Jentsch, 1992; George, Crackower, Abdalla, Hudson, and Ebers, 1993), and mutations of the  $\alpha$  subunit of the dihydropyridine (DHP) receptor have been found to cause hypokalemic periodic paralysis (Jurkat-Rott, Lehmann-Horn, Elbaz, Heine, Gregg, Hogan, Powers, Lopic, Vale-Santos, Weissenbach, and Fontaine, 1995; Ptacek, Tawil, Griggs, Engel, Layzer, Kwiccinski, McManis, Santiago, Moore, Fouad, Bradley, and Leppert, 1995).

Because of their crucial role in generation of action potentials in excitable tissues, the voltage-sensitive sodium channels have held the rapt attention of biologists for decades. Mapping the regions involved in channel activation, inactivation, and ion selection has been an intense area of channel research, yet much remains poorly understood. To date, research on these processes has been largely acquired through the formulation of theoretical models, with subsequent experimental testing of these models. A particularly informative recent experimental strategy has been the theoretical deduction of the structure of voltage-sensitive channels

based upon their primary sequences, and the experimental functional testing of channel proteins containing amino acid substitutions by *in vitro* expression and electrophysiological recording.

An alternative approach is to empirically identify organisms with functional deficiencies of motor response or movement, and identify the corresponding gene and protein. The advantage of this approach is that the effect of protein dysfunction is seen *in vivo*. Recent advances in studying molecular basis of inherited myotonic disorders have facilitated the use of human and animal disease as tools for the understanding of basic biological processes, such as structure/function relationships. Through this work, we have shown that the identification and characterization of genes disrupting motor response or movement directly address relevant human diseases, and also address questions of basic biology.

Here we compare a series of patients with consecutive amino acid replacements of arginine in the extracellular surface of domain IV, transmembrane segment 4. The extreme temperature sensitivity of a proline substitution in this position suggests that this region is important for the structural integrity of the channel, and that cold temperatures may alter the stability of the normal structure.

## Methods

Genomic DNA was isolated from peripheral blood leukocytes (Higuchi, 1989), and each of the 24 exons of the adult skeletal muscle sodium channel  $\alpha$  subunit (SCN4) gene were scanned for possible mutations using PCR-SSCP as previously described (Baquero, Ayala, Wang, Curless, Feero, Hoffman, and Ebeid, 1995). PCR products showing aberrant conformers were cloned into M13 vector and sequenced from both directions (Rojas et al., 1991). Ligase chain reaction (LCR) was performed as previously described (Feero, Wang, Barany, Zhou, Todorovic, Conwit, Galloway, Hausmanowa-Petrusewicz, Fidzianska, Aratiata, Wessel, Sillen, Marks, Hartlage, and Hoffman, 1993).

## Patients

### R1448P: One Patient Was Studied

**Patient VD1.** This eight-year-old girl has been with a foster mother since the age of 3. It was already noted at that time that she had a tendency to walk on her toes and had marked difficulty with walking in the cold, which also produced spasm on closure of her eyelids with delayed opening, particularly when exposed to cold. She was so cold sensitive that even a tepid bath could produced marked muscle spasm. There was at times also slurring of her speech. Cold also induced at times a feeling of being floppy or weak but this was difficult to quantify in terms of severity or duration and there were no overt attacks of paralysis. She also experienced some stiffness on walking in the morning.

On clinical assessment here was no demonstrable voluntary myotonia of the hands, but delayed opening of the eyes after tight closure (lid lag), and marked percussion myotonia of the tongue after a finger tap. Her muscle power was good and she was able to rise from the floor without difficulty. There were a few spontaneous myotonic discharges on electromyography. A diagnosis of paramyotonia congenita was made, with the possibility of an associated mild periodic paralysis.

She has a brother who is clinically normal and two sisters, possibly by a different father, who are said to be normal. There is little information on the biological parents, but the father may have had a Raynaud's phenomenon of the hands in the cold.

#### R1448H: Three Patients from Two Unrelated Families Were Studied

**Family K1800.** Published clinical data (Riggs, Griggs, and Moxley, 1977) was available for an affected 25-year-old male who was subsequently found to have the R1448H mutation. This patient reported nocturnal attacks of weakness which were noted in the early morning since he was 12. The weakness improved with activity. A 5- to 10-min exposure to cold induced marked myotonia, with subsequent dramatic decrease in strength over the next 20 min. Attacks were not induced by rest after exercise. Challenge with potassium ingestion (9 g) caused a strong increase in eye lid myotonia and slight increase in grip myotonia, but no episode of weakness. The patient showed hypertrophied calves and fasciculations (spontaneous myotonia) of muscles. Administration of acetazolamide (250 mg every 6 h for 2 d) abolished myotonia, but increased weakness.

**Family PC18.** This 36-year-old male and his son (three-years old) reported that opening of the eyelids is difficult, in particular after a strong closure (e.g., when sneezing) or repeated closures. Worsening of myotonia with continued exercise (i.e., paradoxical myotonia) was also present in other muscles after cooling. On intensive exercise and cooling, the stiffness was followed by weakness. Spontaneous attacks of weakness were not reported. On examination, the patient showed hypertrophy of the proximal muscles. Myotonia was present in eyelids, tongue, and limb muscles, but percussion myotonia only in the thumb. Electromyography revealed myotonic activity.

#### R1448C: Three Patients from Three Unrelated Families Were Studied

**Family PC2.** Published clinical data was available for seven out of nine family members carrying the mutation (=family D [Ricker, Haass, Rudel, B'hlen, and Mertens, 1980; Ricker, B'hlen, and Rohkamm, 1983]). All showed myotonia in the second decade that was present at all times, though rarely incapacitating. The myotonia was most evident after rest, and would improve with activity. The myotonia was greatly exacerbated by even minor cooling of the muscle, with flaccid paralysis after. Two patients noted exacerbation of myotonia by pregnancy. Cold-induced myotonia was present at a younger age than the symptomatic myotonia in a warm-environment. All affected family members reported spontaneous attacks of generalized weakness which was most pronounced in the legs, and would last from hours to days. When measured serum potassium was increased during these attacks which often occurred in the morning (i.e., hyperkalemic periodic paralysis). Ingestion of potassium (120 mmol KCl by mouth) did induce such attacks of weakness. Tocainide was effective in both reducing myotonia and preventing cold-induced weakness whereas acetazolamide prevented episodes of hyperkalemic weakness (Ricker et al., 1980). Serum creatine kinase was slightly above the upper limit of normal range in all affected family members.

**Family PC33.** This 51-year-old male and his son (27-years old) reported cold-induced stiffness of face and hands, e.g., stiff tongue when eating ice cream. Occasionally, the stiffness gave way to weakness and the cooled muscles may remain

paralyzed for several hours after rewarming. Both experienced spontaneous attacks of weakness, especially of the legs during rest after exercise. All other affected family members were reported to have the same combination of cold-induced symptoms and spontaneous attacks of weakness. Physical examination showed normal muscle relaxation and no percussion myotonia at warm environment but stiffness of muscles after cooling. The index patient revealed slight muscle atrophy and paresis in the shoulder girdle and in the lower legs.

**Family K1637.** This 49-year old female reported episodes of weakness beginning ~8 yr of age, and sensitivity to cold temperatures beginning before age 5 yr. Episodes of weakness were experienced after rest, usually during the day after sitting, and lasted 1-4 h, and were often accompanied by pain. Activity could ward off attacks. The patient complained of urgent urination during attacks. She complains that her "tongue gets big" when ingesting cold drinks or food, and myotonia of the hands in cold water lasting 30-40 min. The patient reported that carbohydrate intake seemed to ablate attacks. Pregnancy did not exacerbate attacks, and in fact may have abated them. Muscle biopsy showed formation of intracellular vacuoles after cooling of the muscle before biopsy. The patient reported that emotional stress, hunger, and exercise all seemed to promote attacks of weakness more often than low temperatures.

**Family K2125.** This 44-year old male reported that his parents noted eyelid myotonia from infancy, and subsequently showed myotonic contractures of eyelids, mouth, hands, and feet upon exposure to cold temperatures. He tells an amusing story of his family members at family reunions eating ice cream and "locking up their entire faces." He reports that episodes of weakness are more debilitating than the cold-induced myotonia, which often occurred in the morning. His most severe attack was during a cold bath after strenuous sports activity, where he fell asleep only to awaken with labored breathing and complete limb paralysis. He was able to feebly call for help, and was rescued from sinking into the water by his brother. The frequency of attacks of weakness increased with age, with two attacks per month with weakness persisting for 3-4 d.

## Results

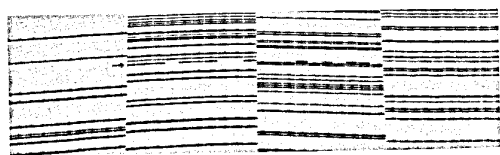
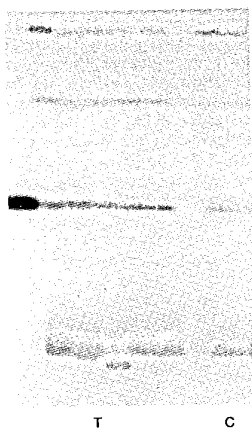
### Identification of Arg1448Pro Mutation

The patient with severe cold induced general weakness (VD1) was tested for two common mutations of the sodium channel gene by LCR (Fecero et al., 1993). This patient did not have these previously identified HyperPP sodium channel mutations. To detect possible unknown sodium channel mutations, we scanned the entire sodium channel gene (24 exons) with PCR-SSCP analysis. A unique conformer in exon 24 of this patient's sodium channel gene was detected (Fig. 1A). The PCR product, which corresponds to amino acid 1429-1518, was then cloned into M13 vector, and 10 clones of this patient's PCR product were sequenced. Sequence analysis showed a C to G substitution in 4 of 10 clones (Fig. 1B), which resulted in an arginine to a proline change at amino acid position 1448 (Wang, Rojas, Zhou, Schwartz, Nicholas, and Hoffman, 1992). This Arg1448Pro change is at exactly the same amino acid position, at which two previously identified PC mutations (Arg1448His and Arg1448Cys) were located (Fig. 2). According to current structure models of the sodium channels, Arg1448 is localized in domain IV, near the extracellular surface of transmembrane segment S4.

**Comparison of Clinical Presentation of Patients with Three Different Mutations at Same Amino Acid Position in Adult Skeletal Muscle Sodium Channel**

The identification of a patient with a novel arginine to proline change (patient VD1) permitted the comparison of a series of paramyotonia patients with different single amino acid changes at position 1448 in the extracellular surface of the sodium

1 2 3 4 5 6 7 8 9



**Figure 1.** Molecular analysis of paramyotonia patient VD1, a patient extremely sensitive to cold temperatures. (A) Shown is SSCP analysis of sodium channel sequence corresponding to part of exon 24 (amino acids 1429-1518) from myotonia patients (lanes 3-9). Six of seven patients showed the normal patterns of conformers (lanes 3, 5-9), whereas patient VD1 (lane 4) showed a unique pattern of SSCP conformers (arrow). Double-stranded and single-stranded PCR products from a normal individual are also shown as controls (lanes 1 and 2).

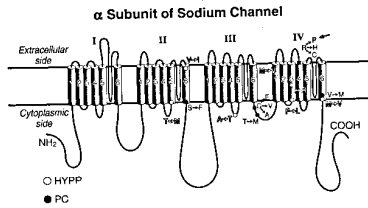
(B) Shown is sequence analysis of the exon 24 SSCP conformers shown in A. The PCR product showing the unique conformers for exon 24 by SSCP was cloned into M13 vector and 10 clones were sequenced. A C to G substitution was found in 4 of 10 clones. This C to G substitution resulted in an arginine to a proline change at amino acid position 1448.

channel. Detailed clinical records were obtained for two unrelated patients with an arginine to histidine change (families K1800, PC18), and four unrelated patients with an arginine to cysteine change (families PC2, PC33, K1637, and K2125) (Table 1). There were similarities between patients with all three mutations: all showed an age of onset which was variable but invariably within the first decade, all showed cold-induced stiffness, and all showed percussion myotonia.

**TABLE 1**  
**Clinical Presentation and Molecular Genetic Data for Patients from Seven Pedigrees**

Mutation	Pedigree	Presenting symptoms	Myotonia		Attacks of weakness and/or paralysis		Frequency	Induced by	Current age	Interictal symptoms
			Spontaneous	Cold	Duration	Hours				
R1448P	PC/VD1	Severe cold-induced stiffness followed by weakness	++	++++	++	++	<1/mo	Low temperature	8	Fixed deformity of ankles and toe walking
R1448H	K1800	Cold-induced stiffness followed by weakness	no	++	+	+	Severe: 1/yr Mild: 1/mo	Rest after exercise, alcohol	25	No
R1448H	PC18	Exercise and cold-induced stiffness followed by weakness	no	++	+	+	No	No	36	Proximal muscle hypertrophy
R1448C	PC2	Muscle stiffness at rest, and greatly exacerbated by cold	++	+++	+	+	<1/mo	Low temperature, potassium	40	No
R1448C	PC33	Cold-induced stiffness of face and hand	+	++	+	+	<1/mo	Rest after exercise, low temperature	51	Slight muscle atrophy and weakness in the shoulder girdle and lower legs
R1448C	K1637	Cold-induced weakness	+	++	+	+	<1/mo	Exercise, emotional stress, hunger, and less frequently cold	49	No
R1448C	K2125	Cold-induced stiffness, exercise, and cold-induced general weakness	+	++	+	+	2/mo (increased with age)	Exercise and low temperature	44	No

The major difference between the three mutations was the increased clinical severity of the proline substitution compared to the histidine and cysteine mutations. The proline substitution is the most dramatic amino acid substitution, both resulting in the loss of a positive charge near the proposed ion conducting pore (S4 segment), and probably causing structural changes in the channel (proline is most often associated with bends in the polypeptide chain). Consistent with the severity of the amino acid change, the patient's clinical phenotype was the most severe of the seven patients studied in detail. This young girl showed extreme sensitivity to cold, with even tepid water causing marked muscle spasms (spontaneous myotonia). She also showed fixed deformity of her ankles with toe walking, suggesting marked myotonia and/or fixed weakness from a young age. Her percussion myotonia was also more dramatic than patients with the other amino acid changes.



mutation described in this report (R1448P), with the two additional amino acid changes found in other patients (R1448C, R1448H). The clinical features of the patients with different mutations at R1448 are compared and contrasted in this report.

Note that some of the mutations shown do not cause "pure" HYPP or PC, and patients with these mutations instead show overlap symptoms or simple myotonia.

## Discussion

### Structure/Function Correlations in the Voltage-sensitive Ion Channelopathies: In vitro Mutagenesis Studies Vs In vivo Patient Studies

With advances in molecular biology and patch clamp recording techniques, in vitro expression analysis has become the method of choice for the study of structure/function relationships in voltage-dependent ion channels. Site directed mutagenesis has been extensively used to produce specific changes in channel primary structure, with the mutant channels then introduced into *Xenopus* oocytes or mammalian cell lines via expression vectors.

Although these in vitro studies have achieved considerable success, they have several limitations. First, these studies are usually directed by hypothetical structural models and may miss functionally important regions not suggested by the models. Second, the expression systems used are oocytes or transformed cell lines: these experimental systems undoubtedly have dramatically different intracellular and membrane environments as compared to the sodium channel's natural host, myofibers or neurons. The differences include presence of the different subunits, membrane structure, tissue-specific posttranslational modifications, among others. Our

**Figure 2.** Summary of sodium channel mutations identified to date. Shown are the single amino acid changes of the adult skeletal muscle sodium channel protein that have been identified in hyperkalemic periodic paralysis (HYPP) and paramyotonia congenita (PC) patients. The hollow arrow at the top of the diagram indicates the position of the arginine to proline

studies of the molecular basis of ion channelopathies in humans and horses have provided an important complementary approach to channel structure and function by studying channel dysfunction in vivo.

Voltage-dependent activation and inactivation are the key functional features of the voltage-dependent sodium channels. Voltage-dependent activation controls the opening of the sodium channel in response to initial depolarization. Inactivation shifts the channel from an open, ion-conducting state to a closed, inactivated state during a sustained depolarization. Mapping the regions involved in activation and inactivation has been one of major focuses of in vitro structure/function studies.

One model of sodium channel inactivation recently supported by extensive data from in vitro structure/function studies (Catterall, 1992) proposes that voltage-dependent activation or opening of the channel occurs because of movement of positively charged S4 segments within the membrane. Thus, selected amino acid substitutions in the S4 segments either shift the voltage dependence of Na channel activation, or alter its steepness; for this reason, these positively charged S4 helices have been considered to be voltage sensors. In this model, rapid or fast inactivation is attributed to movement of the III-IV cytoplasmic loop into the pore (West, Patton, Scheuer, Wang, Goldin, and Catterall, 1992). This "inactivation gate" is believed to interact with docking sites within the cytoplasmic end of the channel pore. Antibodies against this region and single amino acid substitutions within this region can abolish inactivation or slow its rate (Vassilev, Scheuer, and Catterall, 1988, 1989; Scheuer, Wang, Goldin, and Catterall, 1992). The location and identity of the putative docking sites have not been identified. Data from studies of both  $K^+$  and  $Na^+$  channel suggests there may also be a slow component of inactivation which, by contrast with fast inactivation, is not mediated by the III-IV loop (Hoshi, Zagotta, and Aldrich, 1991; Choi, Aldrich, and Yellen, 1991; Zagotta, Hoshi, and Aldrich, 1990). Rather, slow inactivation probably depends primarily on changes in the conformation of regions of the channel at the extracellular face of the membrane.

Do the mutations detected in myotonia and periodic paralysis patients occur in amino acids previously defined as functionally important by biophysical studies? The few mutations causing hyperkalemic periodic paralysis tend to arise in amino acid residues located within the membrane; one exception is a residue near the extracellular face of S4. It is less easy to understand how these affect inactivation. One possibility is that those located near the cytoplasmic surface affect potential docking sites for the inactivation gate, destabilizing or inhibiting inactivation: consistent with this hypothesis is the location of three hyperkalemic periodic paralysis mutations at the intracellular face of the plasma membrane. The location of the three III-IV loop mutations causing myotonia of varying severities correlates well with the purported function of this region as an "inactivation gate." Indeed, the mutations disrupt a "gly-gly- pair proposed to be a hinge for pivoting of the loop.

The amino acid changes causing paramyotonia congenita are diversely situated in the channel. Some are located in either the III-IV cytoplasmic loop or superficially at the extracellular end of S4, including the novel arginine to proline substitution identified in this report (R1448H,C,P) (Fig. 2). While the effect of the III-IV cytoplasmic may be easily explained as an impaired inactivation gate, the functional significance of the S4 mutations, one of which is a novel nonconservative change described in this report (R1448P) is less clear. Recent electrophysiological studies on

R1448H/C sodium channels expressed in a mammalian cell line, performed with patch clamp methods, revealed that this arginine residue is important for fast inactivation (Cattine, George, Zhou, Ji, Sun, Barchi, and Horn, 1994). The authors reported altered channel kinetics and proposed a model in which inactivation is uncoupled from activation. Under the experimental conditions of voltage clamped membrane patches, low temperature had no direct effect on the expressed mutant sodium channels. The novel proline mutation identified here has not yet been studied by patch clamping. Proline is known to have a dramatic influence on the secondary structure of polypeptides. Also, the normal amino acid in this position has a positive charge (arginine) whereas proline carries no charge. Thus, the R1448P changes the charge of this highly conserved region, in addition to having a potentially dramatic effect on the secondary/tertiary structure of the channel. The remarkable sensitivity of this abnormal channel to temperature is more easily rationalized by structural effects of the proline rather than charge effects.

Many other questions remain concerning the functional effects of these mutations. Perhaps most challenging is an explanation of why extracellular  $K^+$  ions can induce the failure of inactivation thought to underlie the pathogenesis of either myotonia or inexcitability and paralysis. In any case, it remains very likely that the analysis of mutations causing these diseases has identified critical regions of the  $Na^+$  channel not previously thought to be functionally important for channel activation or inactivation.

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