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Introduction

Voltage-gated ion channels, ion-conducting proteins equipped with a membrane-spanning pore, gates and voltage sensors, are present in the surface and inner membranes of all excitable and of most non-excitable cells.

The membranes of nerve and muscle cells, the paradigms of excitable cells, are equipped with sodium, potassium, and calcium channels which, by virtue of their voltage sensitivity, allow them to generate action potentials. Conducted along the cell surface, these `spikes' are the bits of information conveyed from cell to cell in the nervous system; in muscle cells, they induce contraction. In vertebrate skeletal muscle, additional voltage-gated chloride channels are responsible for 3/4 to 4/5 of the membrane conductance at rest, thus contributing to a fast repolarization phase of the action potentials.

Non-excitable cells, such as pancreatic islet cells or lymphocytes, also control their specific functions of secretion and immune activity, respectively, by voltage-gated ion channels. In contrast to excitable cells, however, control of function is exerted by small or slow alterations of the resting potential.

The excitation process is one of the basic principles brought to perfection by Nature in the development of the animal kingdom. Since the essential domains of the various ion channels as its mediators were highly conserved for more than 600 million years, these molecules were long imagined to be so sophisticated that mutations in the corresponding genes would not be compatible with life. This idea had to be abandoned when Fontaine et al. (1990) discovered that hyperkalemic periodic paralysis, a hereditary human muscle disorder characterized by episodes of weakness of the skeletal musculature, is linked to *SCN4A*, the gene encoding the . subunit of the adult skeletal muscle sodium channel. Soon afterwards, two further hereditary muscle diseases, paramyotonia congenita (Ebers et al. 1991; Koch et al. 1991; Ptácek et al. 1991b) and

potassium-aggravated myotonia (Lerche et al. 1993; Ptácek et al. 1994b; Ricker et al. 1994), were found to be linked to the same gene. Hyperkalemic periodic paralysis is also known in the horse and is caused by a mutation in the corresponding equine gene (Rudolph et al. 1992b). Moreover, two other hereditary muscle diseases characterized by defective excitation are linked to other ion channel genes. Firstly, myotonia congenita, already described more than 100 years ago (Thomsen 1876) is caused by various mutations in *CLCN1*, the gene coding for the major skeletal muscle chloride channel (Koch et al. 1992) and several mouse models of the disease, in particular the myotonic *adr* mouse, are caused by mutations in the corresponding murine gene (Steinmeyer et al. 1991a). Secondly, hypokalemic periodic paralysis, the classical form of human hereditary periodic paralysis, is linked to *CACNL1A3*, the gene encoding the .1 subunit of the dihydropyridine receptor, the voltage-gated skeletal muscle L-type calcium channel (Fontaine et al. 1994).

In addition to these hereditary `channelopathies' that all involve skeletal muscle channel genes, there are also diseases of the heart and the central and peripheral nervous system, which are based on mutations of genes coding for voltage-gated ion channels. Long QT syndrome type 3, an inherited cardiac arrhythmia in man, is linked to *SCN5A*, the gene encoding the . subunit of the heart muscle sodium channel (Jiang et al. 1994), and a `motor endplate disease' mouse mutant is linked to *Scn8a*, a sodium channel gene expressed in brain and spinal cord (Burgess et al. 1995). Furthermore episodic ataxia type 1 (EA-1), a paroxysmal cerebellar disease associated with myokymia, is caused by mutations in the gene encoding a voltage-gated potassium channel (Browne et al. 1994; 1995).

This review concentrates on naturally occurring mutations affecting voltage-gated ion channels and their pathological consequences. Ligand-gated ion channels and receptors (e.g. the acetylcholine receptor) or transporters such as the one involved in cystic fibrosis, are not considered. Reviews of the structure and function of voltage-gated ion channels in general have appeared for sodium (Catterall 1992; Keynes 1994), potassium (Pongs 1992; Chandy and Gutman 1993), calcium (Hofmann et al. 1994; Melzer et al. 1995) and chloride (Pusch and Jentsch 1994) channels. Reviews on muscle ion channel disorders have also been published (Barchi 1995; Hoffman et al. 1995; Hudson et al. 1995). Extensive descriptions of the clinical features of the muscle channelopathies, including therapeutical considerations, are contained in chapters 49 (Rüdel et al. 1994) and 50 (Lehmann-Horn et al. 1994) of the 2nd edition of the textbook *Myology*.

Sodium channelopathies

The multigene family encoding voltage-gated sodium channels

The human genome contains a number of almost identical genes coding for slightly different voltage-gated sodium channels. The different channel genes are specific for expression in the various tissues and this specialization may help to restrict the consequence of a mutation to a single type of cell or tissue. The various isoforms of voltage-gated sodium channels are heteromeric proteins consisting of a large, heavily glycosylated . subunit and one or two small ß subunits. In the brain, a non-covalently associated ß1 subunit (36 kDa) and a disulfide-linked ß2 subunit (33 kDa) co-purify with the . subunit, whereas in skeletal and heart muscle, only the ß1 subunit has been identified.

Eight different genes are known to encode . subunits, most of them being expressed in brain, peripheral nerve and muscle. A gene cluster occurs on human chromosome 2q21-24.1 and contains several genes, *SCN1A* (2q24, Malo et al. 1994a), *SCN2A* (2q23-24.1, Ahmed et al. 1992), *SCN3A* (2q24, Malo et al. 1994b) for brain channels (type I, II, III) and *SCN6A* (2q21-23,

George et al. 1992a; 1994a) for heart muscle and myometrium. *SCN7A* has not yet been localized in the human genome, but the product of the homologous murine gene has been detected in mouse glia cells (Potts et al. 1993). *SCN8A* maps to chromosome 12q13 and is a candidate gene for inherited neurodegenerative disease as a deletion in the mouse homolog causes a motor endplate disease (Burgess et al. 1995).

Three genes encode three known isoforms of sodium channels in skeletal muscle which differ in their sensitivity to various toxins. Expression depends on the innervation of the muscle fibers. *SCN4A*, the gene encoding the tetrodotoxin(TTX)-sensitive sodium channel . subunit (hSkM1) of adult skeletal muscle, is located on human chromosome 17q23 (Fontaine et al. 1990; George et al. 1992b). The gene contains 24 exons distributed over about 30 kb. As with many genes, the genomic structure becomes more condensed towards the 3' end, with the last 30 % of the coding sequence appearing in a single exon (George et al. 1992b; McClatchey et al. 1992a; Wang et al. 1992). Intron-exon boundaries are known; primer sets consisting of intron sequences for amplification of all 24 exons by use of PCR are available (George et al. 1993b). The cDNA encodes 1836 amino acids in a single open reading frame. *SCN4A* is only expressed in skeletal muscle and its product is the only sodium channel . subunit detectable in the fully differentiated and innervated muscle. Its function is modified by the ß$_1$ subunit (Makita et al. 1994a).

Two other genes encode skeletal muscle . subunits under certain circumstances. *SCN5A*, located on human chromosome 3p21 (George et al. 1995), is expressed in fetal skeletal muscle (SkM2) and in adult cardiac muscle (HH1; Gellens et al. 1992; Rogart et al. 1989) and its gene product is characterized by low TTX sensitivity. *SCN6A*, located on chromosome 2q21-23 (George et al. 1994a), is expressed in fetal skeletal muscle, in cardiac muscle, and myometrium (Gautron et al. 1992).

A single gene on human chromosome 19q13.1, *SCN1B*, encodes the ß1 subunit expressed in brain, heart, and skeletal (ß1=ß) muscle (McClatchey et al. 1993; Makita et al. 1994a,b). Molecular information on the ß2 subunit is now available (Isom et al. 1995a).

Structure and function of hSkM1, the α subunit of the adult human skeletal muscle sodium channel

The subunit hSkM1 is a large glycoprotein of approximately 260 kDa, 25 to 30 % of its mass being carbohydrate (in contrast, the MW of the . subunit expressed in brain, nerve, and in muscle other than adult skeletal is up to 280 kDa). The sequence shows four regions of internal homology, each encompassing 225 to 327 amino acids (Figure 1). These so-called repeats (I to IV) consist of six hydrophobic segments (S1 to S6), putative transmembrane helices located within highly conserved regions in each repeat. Each S4 helix is unique in containing a repeating motif with a positively charged lysine or arginine at every third position. The motif occurs four times in I-S4, five times in II-S4 and III-S4, and eight times in IV-S4. These 22 positive charges are identical in most sodium channels of this gene family and make S4 a natural candidate for the gating voltage sensor (Stühmer et al. 1989). The charges seem to move outward in response to depolarization (Durell and Guy 1992; Yang and Horn 1995), thus playing an essential part in voltage-dependent activation of the channel. Also, most of the hydrophobic residues of the transmembrane segments are conserved.

The carboxy and amino terminals and the linkers joining the repeats are located intracellularly. Their sequences are quite divergent among species as well as channel isoforms, with the exception of the sequence of the linker between repeats III-S6 and IV-S1 to be discussed below. Also most of the short intra- and extracellular loops connecting the transmembrane segments in each repeat are poorly conserved except for the linkers between segments S5 and S6 of each

repeat. These latter linkers consist of an extracellular part and the so-called SS1-SS2 or P-region that dips into the membrane. The highly conserved intramembrane P-regions of the four repeats are thought to form the lining of the channel pore, as in potassium channels (Backx et al. 1992; Heinemann et al. 1992).

A special part of the protein to which a certain function has been assigned, is the above-mentioned intracellular linker connecting repeat III-S6 with repeat IV-S1. Most likely, this mobile chain acts as the inactivation gate of the channel in a way that has been compared with a tethered ball that can plug an opening (Armstrong and Bezanilla 1977). The intracellular orifice of the pore or its surrounding protein parts may act as the acceptor of the ball. In the resting state, the ball is away from the pore and, subsequent to activation, it swings into the mouth to block the ion pathway (similar to N-type inactivation in potassium channels).

The high conservation of the transmembrane segments and of parts of the linker between III-S6 and IV-S1 suggests that the exact amino acid sequence of these parts is essential for proper channel function.

Structure-function relations of the α subunit discovered by artificial site-directed mutagenesis and functional expression

The first experimental studies of the structure-function relations of the channel protein were performed by producing base exchanges in the cDNA at sites of special interest. Functional studies of heterologously expressed mutant channels, performed with a patch-clamp, led to the following results:

(i) The TTX-binding site is in the 5' region of the S5-S6 interlinker of repeat I. This was shown by creation of chimeras between the TTX-sensitive SkM1 and the TTX-insensitive SkM2 (Backx et al. 1992; Chen et al. 1992; Satin et al. 1992). An aromatic amino acid (Tyr or Phe) in the C-terminus of the S5-S6 loop produced the sensitive phenotype, a cysteine the insensitive phenotype.

(ii) The pore has a typical conductance and is highly selective for sodium although it is structurally similar to the pores of the voltage-gated potassium and calcium channels (Noda et al. 1989; Yellen et al. 1991). Mutations in the 3' portions of the S5-S6 interlinkers of each repeat reduced the channel conductance suggesting that portions of all four interlinkers contribute to the lining of the pore (Terlau et al. 1991). The substitution of glutamates for lysine and alanine of the P-region converted the channel to be selective for calcium (Heinemann et al. 1992).

(iii) The hypothesis of a voltage sensor function of the S4 helices characterized by their high charge density was confirmed since reduction of the number of positive charges decreased the voltage dependence of channel activation (Stühmer et al. 1989). However, replacement of certain neutral amino acids altered activation in a similar way suggesting a complex interaction during activation by charge movements rather than a simple dependence on the net charge of a dipole.

(iv) The ball-and-chain model (Armstrong and Bezanilla 1977), originally developed to explain the abolition of sodium channel inactivation following enzymatic removal or functional blockage of the cytosolic gate (Armstrong et al. 1973; Vassilev et al. 1988; 1989) and later verified for the N-terminal of the fast inactivating potassium channel (Hoshi et al. 1990; Patton et al. 1993), had to be modified on account of the mutagenesis experiments. Deletions in the N-terminus part of the linker between repeats III and IV, i.e. the inactivation gate, removed inactivation whereas the positively charged particles, the supposed `ball', were not required for proper inactivation (Stühmer et al. 1989; Moorman et al. 1990). However, replacement of three hydrophobic amino acids (Ile-Phe-Met by Gln-Gln-Gln) in the same part of the linker completely eliminated inactivation (West et al. 1992). The single substitution (Phe-1489-Gln in the

rat brain type IIA sodium channel, corresponding to phenylalanine 1311 of hSkM1) alone abolished fast inactivation (West et al. 1992). Hydrophobic parts of the intracellular orifice of the pore or its surrounding protein parts may act as acceptor of the hydrophobic motif. In the closed resting state, the lid is away from the pore, and following activation the lid moves into the mouth to inactivate the channel.

Structure and function of the ß subunits and their effects on the α subunit

The ß1 subunit is a small glycoprotein of 38 kDa, containing 218 amino acids in the rat. Sequence analysis suggests a single transmembrane segment with an extracellular N-terminus containing glycosylation sites (Isom et al. 1992). The human and rat cDNAs exhibit 96 % homology (McClatchey et al. 1993). The ß and α subunits interact in a 1:1 stoichiometry by a non-covalent binding. Perhaps ß binds to only one of the four repeats (Kraner et al. 1985). The structure of ß2 which is covalently attached to the α subunit (Hartshorne et al. 1982) by disulfide bonds has just been clarified (Isom et al. 1995a).

Injection of the cRNA encoding hSkM1 into *Xenopus* oocytes results in the expression of functional sodium channels (Trimmer et al. 1989). However, the inactivation of the macroscopic sodium current conducted by these channels is slower than that of native channels or channels produced by co-injection of total mRNA or the α subunit cRNA (Krafte et al. 1988; 1990; Isom et al. 1992; Cannon et al. 1993b). This slowed inactivation is caused by channel re-openings occuring as single events or as occasional bursts (ten to hundreds of repetitive openings). It occurs much more frequently with the isolated α subunit. The occurrence of various gating modes in a single population of channels seems to reflect the existence of at least two conformations of the α subunit. The physiologically preferred gating mode (no re-openings) can be stabilized by co-expression with the ß1 subunit (Isom et al. 1994; 1995b; Ji et al. 1994; Schreibmayer et al. 1994) which accelerates inactivation in *Xenopus* oocytes by an extracellular interaction (Chen and Cannon 1995). In addition, the ß1 subunit increases functional expression of the α subunit resulting in a larger current amplitude. It shifts the steady-state activation and inactivation curves of rat and human sodium channels expressed towards more negative potentials (about 5 to 10 mV), and accelerates recovery from inactivation (Wallner et al. 1993; Makita et al. 1994a), whereas it had no effect on the gating of the human heart sodium channel (Makita et al. 1994a). Co-expression of α1 in mammalian cells without endagenous α1 production has similar effects except for the almost missing acceleration of the already fast inactivation of the channel (Isom et al. 1995b). Different protein glycosylation is discussed to be responsible for the slow inactivation of the isolated α subunit in oocytes.

Human diseases due to mutations in *SCN4A*

As soon as *SCN4A* was cloned, a genetic approach was available to test the hypothesis of primary sodium channel defects in some hereditary myotonias and periodic paralyses. Abnormal inactivation of sodium currents had been the major result of electrophysiological studies performed on excised muscle specimens of patients having paramyotonia or periodic paralysis (Lehmann-Horn et al. 1987a,b). Linkage of hyperkalemic periodic paralysis to *SCN4A* (Fontaine et al. 1990) provided further evidence for the existence of a sodium channel disease in man. Three groups then showed independently that paramyotonia congenita is also linked to *SCN4A* (Ebers et al. 1991; Koch et al. 1991; Ptácek et al. 1991b; see Rüdel et al. 1993). Finally, molecular biology revealed that the myotonia in various families that were previously diagnosed as having `abnormal forms' of myotonia congenita (which is now known to be a chloride channel disease, see below) is in fact the consequence of mutations in *SCN4A*. Several clinical variants of such `sodium channel myotonias' (myotonia fluctuans, Ricker et al. 1990; 1994; Lennox et al. 1992),

myotonia permanens (Lerche et al. 1993), acetazolamide-responsive myotonia (Ptácek et al. 1994b) have been described that are clinically different from classical hyperkalemic periodic paralysis or paramyotonia congenita. All types of sodium channel myotonia differ distinctly from the chloride channel myotonias, in that ingestion of potassium by the patient enhances the myotonia (without leading to weakness, as would be characteristic for hyperkalemic periodic paralysis). Therefore, the sodium channel myotonias are grouped together as `potassium-aggravated myotonias'.

All sodium channel diseases have in common that they are transmitted with dominant mode of inheritance and that the basic pathology may include both hyper- and hypo-excitability in the same patient at different times. Fortunately for the patients, the corresponding symptoms, muscle stiffness (myotonia) and muscle weakness (paralysis), are not present all the time. Rather, they are elicited by typical stimuli. A typical trigger for an episode of weakness in hyperkalemic periodic paralysis would be rest after a heavy work load; stiffness and weakness in paramyotonia congenita is triggered by muscle exercise during exposure of the muscles to cold, and ingestion of potassium-rich food may induce muscle stiffness in patients having myotonia fluctuans. Even more fortunately for the patients, all these symptoms disappear spontaneously within an hour or so. Nevertheless, the episodes hamper the patient's life considerably, although they may be prevented to a certain extent by proper behavior and symptomatic treatment with drugs (for review see Lehmann-Horn et al. 1994).

Hyperkalemic periodic paralysis. The disease was first described by Tyler et al. (1951) and was extensively investigated by Gamstorp (1956) who clearly differentiated it from `paroxysmal familial paralysis' and named it `adynamia episodica hereditaria'. Clinically, the most striking difference of the two diseases is that, during the paralytic episode, serum potassium decreases in the former and increases in the latter. To stress this distinction, the names hypokalemic periodic paralysis and hyperkalemic periodic paralysis are now preferred for these two diseases. It should be mentioned that although the respective mutations concern different channels, the symptoms associated with these two diseases may be so similar that, before the era of molecular biology, differential diagnosis of patients was sometimes very difficult.

Hyperkalemic periodic paralysis has three distinct variants. It can occur (i) without myotonia, (ii) with clinical or electromyographic myotonia, or (iii) with paramyotonia. In some patients, a chronic progressive myopathy may develop (Bradley et al. 1989) which seems to be caused by a single mutation (Ptácek et al. 1991a; Lehmann-Horn et al. 1993). A few families have been reported in which a change of the serum potassium was barely detectable during the episodes of paralysis (Poskanzer and Kerr 1961). Such **normokalemic periodic paralysis** should probably be regarded as a special condition of hyperkalemic periodic paralysis (Lehmann-Horn et al. 1993).

The diagnosis is based on the presence of typical attacks of weakness or paralysis, the positive family history, and the myotonic or paramyotonic phenomena, if present. Except for some older patients with progressive myopathy, the muscles are well-developed.

An elegant provocative method, often used in experiments designed to study the pathophysiology of paralytic attacks, consists of exercise on a bicycle ergometer for 30 min so that the pulse increases to 120 to 160 beats/min, followed by absolute rest in bed (Ricker et al. 1989). The serum potassium level rises during exercise and then declines to almost the pre-exercise value, as in healthy individuals. Ten to 20 min after the onset of the bed rest, a second hyperkalemic period occurs in the patients but not in normal subjects. It is during this period that the patients become paralyzed.

Paramyotonia congenita. The hallmarks of this disease, as first described by Eulenburg (1886) and later confirmed in many families by Becker (1970) are: (i) paradoxical myotonia, defined as myotonia that appears during exercise and increases with continued exercise, (ii) severe worsening of the exercise-induced myotonia by cold, (iii) a predilection of the myotonia for the face, neck, and distal upper extremity muscles, and (iv) weakness after prolonged exercise and exposure to cold.

Percussion myotonia is not pronounced, but many patients exhibit the lid lag phenomenon and in the course of repeated strong contractions of the orbicularis oculi the eyelids open more and more slowly. In a number of families the symptoms are clearly different from those found in most cases of paramyotonia:

(i) Some patients experience myotonic stiffness during work even under warm conditions. Such patients require long-term medication.

(ii) In some kinships cold induces stiffness but no weakness (Koch et al. 1995)

(iii) Still other patients are immediately paralyzed by cold.

(iv) In some kinships, the patients have not only paramyotonic symptoms, but also temperature-independent paralytic attacks, resembling those in hyperkalemic periodic paralysis. The attacks usually begin early in the day and can last for several hours. Oral intake of potassium can induce such attacks in these patients distinguished by `paramyotonic hyperkalemic periodic paralysis'.

These special forms led to the suggestion that hyperkalemic periodic paralysis and paramyotonia congenita are two facets of the same disease (DeSilva et al. 1990). On the other hand, the symptoms in the `pure' forms are so different that it seems reasonable to keep both nosological entities, although it is now known that the two diseases are allelic.

Permanent weakness and muscle atrophy are not signs of paramyotonia congenita. The electromyogram always shows myotonic discharges in all muscles, even at a normal muscle temperature. The serum creatine kinase is often elevated, sometimes to 5 to 10 times above normal.

Potassium-aggravated myotonia. For many families with dominant myotonia thought to have a subtype of Thomsen's disease, a muscle chloride channel disease, molecular genetics revealed mutations in SCN4A. In contrast to Thomsen's disease, these patients developed severe stiffness following oral ingestion of potassium. The spectrum of the degree of myotonia is large, ranging from the mild myotonia fluctuans to the very severe myotonia permanens.

Myotonia fluctuans and acetazolamide-responsive myotonia. The clinical signs of myotonia fluctuans resemble those of myotonia congenita (see below) with the peculiarity that the stiffness tends to fluctuate from day to day. The patients never experience muscle weakness and are not very sensitive to cold as regards muscle stiffness. Their muscle stiffness is provoked by exercise: usually it occurs during rest about half an hour after the exercise and lasts approximately another hour. Ingestion of potassium aggravates myotonia but does not induce weakness as in hyperkalemic periodic paralysis. Also other depolarizing agents such as suxamethonium can induce or aggravate myotonia so that severe ventilation problems may occur during general anesthesia. The incidence of such events is high in myotonia fluctuans families (Ricker et al. 1994; Iaizzo and Lehmann-Horn 1995; Vita et al. 1995), and there seems to be no other biological reason for this than the frequent absence of clinical myotonia in these patients. Thus, the anesthesiologists are unaware of the condition. Therefore it is worth mentioning that even during the spells of absence of clinical myotonia, latent myotonia can be consistently recorded by the use of electromyography. In acetazolamide-responsive myotonia (Trudell et al. 1987),

also described as atypical myotonia congenita (Ptácek et al. 1992b), the muscle stiffness also fluctuates and, in addition, muscle pain is induced by exercise. Both stiffness and pain are alleviated by acetazolamide.

Myotonia permanens. The definition of this disease is the consequence of genotyping a patient earlier thought to have a `myogenic' type of Schwartz-Jampel syndrome (Spaans et al. 1990); in fact he was carrying a mutation in *SCN4A* (Lerche et al. 1993; Mitrovic et al. 1995). Continuous myotonic activity is detectable in the electromyogram of these patients causing persistent severe myotonia. Muscle hypertrophy, particularly in the neck and shoulder, is very marked. During attacks of severe muscle stiffness the patients suffer from impaired ventilation due to stiffness of the thoracic muscles. In particular, children can suffer from acute hypoventilation and this may lead to cyanosis and unconsciousness, so that such episodes were in the past mistaken for an epileptic seizure. In spite of the misdiagnosis, anti-epileptic medication, e.g. administration of carbamazepine, was successful in these cases because of its antimyotonic effects. The patients could probably not survive without persisting treatment. The aggravation of myotonia by potassium was proven in vitro (Lerche et al. 1993). Since the myotonia is so severe in these patients, potassium must never be administered as a diagnostic tool.

A further indication of the severity of this disease is that the few patients known to date were sporadic cases having a *de novo* mutation, i.e. their proven biologic parents did not carry the mutation; none of these patients has offspring.

Quarter horse hyperkalemic periodic paralysis due to a mutation in *Scn4a*

An equivalent condition to human hyperkalemic periodic paralysis in man has become known in the Quarter horse, a very common breed of race horses in the USA (Cox 1985; Pickar et al. 1991). The symptoms are similar to those described above for the human disease, but the condition seems to be sometimes more serious than in man as some affected horses have died from attacks as a result of shock or ischemia. The disease, the most frequent hereditary disorder of horses, is linked to the equine homologue *Scn4a* (Rudolph et al. 1992a,b).

Since the discovery of this homology, affected horses have been used for the study of the cellular and physiological factors dictating the onset and severity of attacks and the relationship between exercise, systemic potassium, catecholamines, and other factors influencing muscle metabolism. Thus, the hyperkalemic horse has been used to show the first correlation of levels of mutant mRNA relative to normal mRNA as a likely determinant of clinical severity in dominantly inherited disease (Zhou et al. 1994).

Pathophysiology of sodium channel diseases

Electrophysiology on excised muscle specimens from such patients revealed a non-inactivating component of the sodium current as a specific abnormality (Lehmann-Horn et al. 1983; 1987a,b; 1991; Cannon et al. 1991). This indicated that both key symptoms, stiffness and weakness, are caused by the same mechanism, namely a long-lasting depolarization of the muscle fiber membranes. When the depolarization is mild, say 5 to 10 mV, this may fulfil exactly the condition for the voltage-dependent sodium channels to open again after an action potential, i.e. for repetitive firing which is the basis for the involuntary muscle activity that the patient experiences as muscle stiffness. This hyperexcitable state can be computer-simulated (Cannon et al. 1993a) and mimicked by anemone toxin (Cannon and Corey 1993). When the depolarization is strong, say 20 to 30 mV, the majority of the intact sodium channels adopt the state of inactivation, i.e. the muscle fibers become inexcitable, which is the basis of the muscle weakness. When all fibers of a muscle are inexcitable, this results in complete paralysis (for

unknown reason, the diaphragm is spared, and fortunately the heart muscle in which a different gene is expressed).

Naturally occurring mutations in *SCN4A*

With the knowledge of the special function of some domains of the channel protein, it is interesting to see where the various point mutations are situated and how they might produce the different symptoms.

Nineteen disease-causing amino acid changes have been identified to date (Figure 1, Table 1), and 5 additional silent amino acid substitutions were found in healthy controls. Five of the mutations result in hyperkalemic periodic paralysis, 8 in paramyotonia congenita, and 6 in potassium-aggravated myotonias. At first glance, no obvious correlations can be made between the location of amino acid change and the clinical phenotype. However, the distribution of the various substitutions along the protein molecule might display certain systematic features. The number of substitutions increases as one proceeds from the N to the C terminus: none of the substitutions is situated in repeat I, and only 3 substitutions are located in repeat II, all of them in transmembrane segments. Three other substitutions are situated in repeat III, two of them in an intracellular interlinker and one in transmembrane segment 6. The majority of the substitutions are situated in the linker connecting repeats III and IV (n=5) and in repeat IV (n=9).

None of the substitutions concerns an extracellular loop, whereas 7 substitutions concern intracellular loops. Of the 12 substitutions in the transmembrane segments, 6 are situated near the intracellular membrane face, 5 are near the extracellular face, and only one substitution is located in the middle of a transmembrane segment (in IV-S4).

The most common mutation causes hyperkalemic periodic paralysis and predicts Thr-704-Met in segment II-S5. The mutation may cause the myotonic or the non-myotonic form of the disease (Ptácek et al. 1991a; Feero et al. 1993; Wang et al. 1993; Plassart et al. 1994). In either case progressive myopathy is found in older and sometimes even in younger patients. The second most common mutation, also causing HyperPP and predicting Met-1592-Val in IV-S6, is always associated with myotonia and does not lead to permanent weakness (Rojas et al. 1991; Feero et al. 1993; Wang et al. 1993). The rare third and fourth mutations, predicting Ala-1156-Thr in the linker between III-S4 and III-S5 (McClatchey et al. 1992b) and Met-1360-Val in IV-S1 (Lehmann-Horn et al. 1993), respectively, are characterized by incomplete clinical penetrance in females, although `unaffected' family members show electrical myotonia in the electromyogram indicating that penetrance is really 100% (Lehmann-Horn et al. 1993). The fifth mutation, predicting Val-783-Ile, is a sporadic case with hyperkalemic periodic paralysis and cardiac dysrhythmia (Baquero et al. 1995). One family that was convincingly diagnosed as having hyperkalemic periodic paralysis, was not linked to *SCN4A* (Wang et al. 1993). Genetic heterogeneity is the most probable explanation.

Three of the 8 point mutations leading to paramyotonia congenita involve Arg-1448 in segment IV-S4, replacing it by histidine, cysteine or proline (Ptácek et al. 1992a; Wang et al. 1993; Meyer-Kleine et al. 1994a; Wang J et al. 1995; Lerche et al. 1996b). The more the substitution differs from the normal arginine the more severe the clinical symptoms: a patient having the proline change is the most severely afflicted paramyotonia patient observed to date, with fixed ankle joint contractures (probably secondary to myotonia), and induction of severe myotonia and weakness by even tepid temperatures (Wang J et al. 1995; Lerche et al. 1996b).

A frequent paramyotonia-causing mutation results in Thr-1313-Met (McClatchey et al. 1992c; Ptácek et al. 1993; Plassart et al. 1994; Tahmoush et al. 1994) and affects the cytoplasmic loop between repeats III and IV, i.e. the inactivation gate. Other mutations predict Leu-1433-Arg in IV-S3 (Ptácek et al. 1993), Val-1293-Ile in III-S6 (Koch et al. 1995; Heine et al.

1996, Mitrovic et al. 1996), Val-1458-Phe in IV-S4 (Heine et al. 1996), and Phe-1473-Ser in the intracellular loop connecting S4 and S5 of repeat IV (Heine et al. 1996).

Two further mutations, predicting Gly-1306-Glu (Lerche et al. 1993) and Gly-1306-Ala (Lerche et al. 1993; Ricker et al. 1994; Mitrovic et al. 1995; Vita et al. 1995), cause myotonia permanens and myotonia fluctuans, respectively. A third, Gly-1306-Val (McClatchey et al. 1992c; Lerche et al. 1993; Plassart et al. 1994), causes a moderate degree of myotonia. The mutations substitute one of a pair of glycines (Gly-1306/Gly-1307) in the III-S6 to IV-S1 linker and thus affect the putative inactivation gate at the same position as the Gly-1306-Val substitution causing paramyotonia congenita. This pair of unbranched amino acids is believed to act as a kind of `hinge' of the inactivation gate and, therefore, should be essential for proper channel inactivation (West et al. 1992). As discussed above for the three Arg-1448 substitutions, the more the substituted amino acid differs from the normal glycine, the greater the membrane hyperexcitability, and the more severe the clinical symptoms: a change from glycine to glutamic acid, an amino acid with a long and charged side-chain, causes myotonia permanens, the most severe form of all known non-dystrophic myotonic diseases; valine, an amino acid with a side-chain of intermediate size, causes moderate myotonia; whereas alanine, with a short side--chain, results in a benign, often `subclinical' form of myotonia fluctuans.

The other two mutations lead to moderate myotonia, Val-1589-Met in IV-S6 (Heine et al. 1993; Mitrovic et al. 1994), or myotonia fluctuans, Ser-804-Phe in II-S6 (Ricker et al. 1994). A family having mutation Ser-804-Phe had earlier been described as having `features of paramyotonia congenita and myotonia congenita' (McClatchey et al. 1992b).

Finally, a mutation predicting Ile-1160-Val in the linker of segments 4 and 5 of repeat III was found to cause acetazolamide-responsive myotonia (Ptácek et al. 1994b).

Frequency of natural mutations in *SCN4A*

Mutations of human genes may arise either as a consequence of endogenous error-prone processes, such as DNA replication and repair, or as a result of exposure to exogenous factors, e.g. chemical mutagens or ionizing/UV irradiation. A systematic investigation of spontaneous point mutations causing human genetic disease disclosed that the dinucleotide CpG (p denotes cytosin 5' to guanin 3' binding) is a `hot spot' for mutations in the human genome (Cooper and Krawczak 1990). This has been explained to be the result of the hypermutability of methylated CpG, deamination of 5-methylcytosine (5mC) to thymidine in this doublet giving rise to C → T or G → A substitutions depending upon in which strand the 5mC is mutated (Duncan and Miller 1980). Thymidine being a `normal' nucleoside is thought to be less readily detectable and removable by cellular repair mechanisms. Evaluation of 139 point mutations causing human genetic diseases other than sodium channelopathies, and consistent with methylation-mediated deamination of 5mC, yielded 21 CG → TG and 23 CG → CA mutations, i.e. a total of 44 or 31.7 % (Cooper and Krawczak 1990).

Of the 19 mutations detected in *SCN4A*, 8 (> 40%) contained a mutated base in the CpG dinucleotide. Thus, the frequency of CpG mutations in *SCN4A* corresponds well with that found in general for disease-causing point mutations (Heine 1995). With the exception of the "Ravensberg" families (Arg-1448-His, Meyer-Kleine et al. 1994a), a founder effect for the mutations could be excluded (Wang et al. 1993). Considering the number of independently originating *SCN4A* mutations in the 94 non-related families entering this review, 60 concerned the CpG dinucleotide. Thus, the percentage of mutations in a CpG nucleotide in *SCN4A* is even higher, i.e. around 64 % of the total number.

Second in frequency amongst the human disease-causing point mutations (Cooper and Krawczak 1990) were those affecting a GG dinucleotide. Interestingly, mutations of this type

are responsible for the three substitutions for the amino acid Gly-1306-Ala/Val/Glu in the supposed inactivation gate (Heine 1995).

Properties of mutant sodium channels in heterologous expression systems

When wild-type *SCN4A* is transfected into mammalian cells, e.g. human embryonic kidney (HEK293) cells, inactivation is normal (Ukomadu et al. 1992).

Patch-clamp recordings of sodium currents from HEK293 cells transfected with mutant channels revealed that the mutations leading to the clinically different phenotypes of hyperkalemic periodic paralysis, paramyotonia congenita or one of the potassium-aggravated myotonias, yielded similar results: The major defect of all investigated mutants was a more or less impaired inactivation (Cannon and Strittmatter 1993; Cummins et al. 1993; Chahine et al. 1994; Mitrovic et al. 1994; 1995; Yang et al. 1994). The macroscopic changes of inactivation were evident as (i) a slowing of the decay of the current transients, elicited by depolarizing voltage pulses, (ii) an increased steady-state current following the current, (iii) shifts of the steady-state inactivation and/or activation curves increasing the window current, and (iv) uncoupling of inactivation from activation. Single-channel recordings revealed that in particular (ii) was owing to a shift in the equilibrium between two physiological gating modes known for inactivation. A qualitatively similar but quantitative smaller late activity has been also seen with membrane patches from native normal skeletal and heart muscle as well as from brain cells of various species (Patlak and Ortiz 1986; Nilius 1988; Moorman et al. 1990; Zhou et al. 1991; Saint et al. 1992; Ju et al. 1994).

Careful investigation revealed that the changes of inactivation are not quite the same with different mutants. For example, slowing of the current decay was most pronounced with substitutions for Arg-1448 causing paramyotonia (Chahine et al. 1994; Lerche et al. 1996b), whereas a large persistent sodium current was found for the mutants Met-1592-Val (HyperPP, Cannon and Strittmatter 1993) and Val-1589-Met (PAM, Mitrovic et al. 1994).

There is reason to believe that it is hinged-lid inactivation of the channels that is affected by the thirteen amino acid substitutions on the cytoplasmic surface of the channel. Particularly, the mutations affecting Gly-1306 may directly affect the hinge of the inactivation gate (West et al. 1992; Lerche et al. 1993; Mitrovic et al. 1995). In contrast, the mutations within the voltage sensor IV/S4 (Arg-1448-His/Cys/Pro) uncouple activation and inactivation which results in a relatively voltage-independent time course of inactivation (Chahine et al. 1994; Lerche et al. 1996b). Thus, the disease causing mutations in the human muscle sodium channel have uncovered functions of the channel protein which had previously not been recognized in studies of in-vitro mutagenesis.

Under the experimental conditions of voltage-clamp of heterologous cells, neither extracellular potassium nor low temperature had a marked and reproducible effect on any of the mutant channels investigated. Therefore, these triggering factors probably exert their effects indirectly, e.g. they physiologically cause slight and sustained membrane depolarization followed by an increase in the persistent sodium current.

Long QT3 syndrome, a disease linked to *SCN5A* encoding the α subunit of the human cardiac muscle sodium channel

In the congenital disorder of long QT syndrome (LQT), the cardiac action potential is prolonged as clinically evidenced by a prolonged QT interval of the electrocardiogram. The condition is transmitted as an autosomal dominant trait. Patients are predisposed to syncope and sudden death owing to ventricular arrhythmias.

Linkage studies have demonstrated genetic heterogeneity for the disease by the identification of so far three different loci: LQT1 on chromosome 11p15.5, LQT2 on 7q35-36, and LQT3 on 3p21-24. Whilst no candidate gene is known for LQT1, mutations in *HERG* have been claimed to cause LQT2 (Curran et al. 1995). The *HERG* gene product is believed to be a potassium channel conducting the rapidly activating delayed rectifier current (I_{Kr}) in cardiac muscle (Sanguinetti et al. 1995).

LQT3 was shown to cosegregate with polymorphisms within *SCN5A*, the gene encoding the . subunit of the major cardiac sodium channel (Jiang et al. 1994). Analysis of the DNA of affected members of two unrelated LQT3 families revealed an in-frame deletion in *SCN5A* which results in the omission of three highly conserved amino acids (KPQ) in the cytoplasmic linker between repeats III and IV, the inactivation gate (Wang et al. 1995).

Expression of the mutated gene in *Xenopus* oocytes demonstrated abnormal sodium channel gating and, as a result, a small persistent inward current (Bennett et al. 1995). Such a persistent sodium current may also flow during the plateau phase of the action potential of cardiac myocytes. The plateau is normally maintained by a delicate balance between inward and outward currents, and repolarization takes place when the outward currents prevail over the inward currents. The sustained inward current generated by the mutant sodium channel prolongs the action potential and thus lengthens the QT interval. Thus in LQT, the first recognized form of a myocardial ion channel disease, the defect in channel function seems to be very similar to defects described in the skeletal muscle sodium channel diseases.

A mouse mutant having motor endplate disease (MED), linked to *Scn8a*

The disease, transmitted as a recessive trait, is characterized by progressive weakness and wasting of the skeletal muscles. It begins around day 9 after birth at the hind limbs and ends fatally 10-15 days later. The cerebellar Purkinje cells are degenerated and show a reduction in sodium-dependent spontaneous action potentials. The motor neurons have an increased temperature sensitivity of action potentials as well as a reduced conduction velocity and prolonged refractory period (Angaut-Petit et al. 1982). The alterations in the motor nerves probably account for the failures in neuromuscular transmission. A progressive abnormality of the skeletal muscle innervation is marked sprouting. As the muscle weakness becomes more severe, the terminal arborizations become increasingly more complex, in particular more elongated than normal, and may be in contact with several muscle fibers (Duchen 1970). The light microscopic changes are in many ways similar to those seen in muscle after local injection of botulinum toxin.

A gene encoding a sodium channel . subunit was isolated from the flanking region of a transgene-induced allele of the *med* gene and was called *Scn8a*. It is expressed in brain and spinal cord but not in skeletal or heart muscle. *Scn8a* is most closely related to a brain cDNA from the pufferfish *Fugu rubripes*, with 83 % overall sequence identity and several shared insertions and deletions that are not present in other mammalian cDNAs. The divergence of *Scn8a* from the other brain sodium channels thus occurred prior to the separation of fish and mammals and predates the origin of the gene cluster on chromosome 2. A deletion at the transgene insertion site spanning 5 to 10 kb of genomic DNA causes loss of expression (Burgess et al. 1995). In MED mice, i.e. animals that are homozygous for the deletion, the absence of the *Scn8a* gene product could account for the above-mentioned symptoms although a multiplicity of other sodium channel isoforms might be expressed.

Paralysis in *drosophila* due to *para* locus sodium channel mutations

Para encodes a functionally predominant class of voltage-gated sodium channels in neurons of the fruit fly *drosophila melanogaster*. It contains a minimum of 26 exons distributed over more than 60 kb of genomic DNA. The transcript undergoes alternative splicing to produce several distinct channel subtypes. The proteins are very similar to those of vertebrate sodium channels (Loughney et al. 1989).

Several insertions of transposable elements within introns of several *drosophila* genes are known to interfere with transcription or processing of the RNA (Levis et al. 1984). These mutations often allow some degree of expression because a small fraction of the transcripts from the mutated gene is correctly transcribed and processed to produce functional mRNAs. A similar mechanism seems to operate for the *para* insertional mutations, so that they result in a low sodium channel expression rate rather than in the production of altered channels. Thus, in certain *para* mutants, the number of sodium channels may be greatly reduced in neurons whose sodium channels are expressed by the *para* gene. Excitation of these neurons may physiologically still be possible, but may be abortive under adversary circumstances. Indeed, some *para* mutants become paralyzed at elevated temperature, probably because faster channel kinetics cause conduction block (Loughney et al. 1989).

Potassium channelopathies

The multigene family encoding voltage-gated potassium channels

A mutant of the fruit fly *drosophila melanogaster* episodically presents, and consistently responds to ether anesthesia, with jerking leg movements and was therefore named `Shaker'. Electrophysiological investigation of the muscle fibers of this mutant revealed potassium currents that inactivated much faster than those of wild type *drosophila*. This led to the correct assumption that the Shaker mutation is located in a gene coding for a potassium channel (*Shaker* gene). Cloning and functional expression of this gene was the starting point for the identification of a large gene family encoding voltage-gated potassium channels that have homologs in vertebrates. The human homolog of the *Shaker* gene was termed *Kv1.1* or *KCNA1* according to the nomenclature of mammalian potassium channel genes (Gutman and Chandy 1993). Typically enough, the first human channelopathy detected involving a voltage-gated potassium channel is caused by a mutation in *Kv1.1* (Browne et al. 1994).

In vertebrates, the family consists of six subfamilies, with *Kv1.1* to *Kv1.7* (*KCNA1* to *KCNA7*) belonging to the *Shaker* gene-related subfamily 1. Correspondingly, subfamily 2 (*Kv2* or *KCNB*) is related to *Shab*, subfamily 3 (*Kv3* or *KCNC*) to *Shaw*, and subfamily 4 to *Shal*, genes that are all related to potassium channel genes in *drosophila*. Further related mammalian subfamilies encode either voltage-gated (*Kv5*/*KCND*, *Kv6*/*KCNG*) or ligand-gated potassium channels. The voltage-gated potassium channels inactivate at different rates and to a varying extent (fast N-type and slow C-type inactivation). They are found in almost all eukaryotic cells of the animal and plant kingdoms (Rudy 1988) and are not only involved in the excitability of cells, such as nerve or muscle cells, but also of `inexcitable' cells such as lymphocytes and pancreatic islet cells (Grissmer et al. 1990).

Structure of the *drosophila*-related mammalian potassium channels

The functional channels are tetrameric complexes, very similar to the sodium channel. Each potassium channel domain is encoded by a gene, whereas in the sodium channel, the whole tetramer is encoded by one gene. Corresponding to the segments S1-S6 in each of the repeats I-IV of the sodium channel, each potassium channel monomer contains six membrane-spanning .-

helices, and the segments S5 ans S6 and the linkers between them make up the lining of the conducting pore (Pongs 1992; Salkoff et al. 1992). Homo- and heteromultimeric potassium channels may occur in nature, with heteromers belonging to the same subfamily. Most homotetramers, e.g. all *Shaker*-related channels except Kv1.4, inactivate slowly. Heterotetramers show fast inactivation if at least one of the four domains, e.g. Kv1.4, carries the inactivation ball within its N-terminus (MacKinnon et al. 1993).

Episodic ataxia with myokymia (EA-1), a Kv1.1 channelopathy, and EA-2, a possible potassium channelopathy

This autosomal dominant human disease is characterized by episodic failure of excitation of cerebellar neurons and sustained hyperexcitability of the second motoneurons. The latter feature leads to repetitive twitching of small muscle fibers, e.g. those around the eyes or in the hands. These so-called myokymias respond to anticonvulsants such as carbamazepine. The onset of motion and exercise may provoke attacks of atactic gait that last for seconds to minutes. Acetazolamide is able to reduce the number and severity of attacks in some families (Gancher and Nutt 1986; Brunt and van Weerden 1990). Litt et al. (1994) showed linkage of the disease to chromosome 12p13, and six point mutations have been identified in Kv1.1 for six families: Val-176-Phe, Arg-240-Ser, Phe-250-Ile, Val-410-Ala, Glu-325-Asp, Phe-184-Cys (Browne et al. 1994; 1995) (Figure 2).

A clinically similar disease, episodic ataxia-type 2 (EA-2), has recently been linked to a locus that possibly encodes another potassium channel (Vahedi et al. 1995). This form of episodic ataxia is generally associated with interictal nystagmus. Attacks are provoked by emotional stress and exercise but not by starting movements. Attacks last for several hours to more than a day. Some patients develop progressive ataxia and dysarthria and may display cerebellar vermian atrophy on magnetic resonance imaging. Acetazolamide is usually very effective in reducing the frequency of attacks.

Calcium channelopathies

Types of voltage-gated calcium channels

Initially, voltage-gated calcium channels were distinguished on the basis of their inactivation properties and named T-type when conducting transient current or L-type when conducting long lasting current. A later classification noted their presence in certain tissues, naming them B for brain, N for neuronal, and P for cerebellar Purkinje cells. Then, another classification was introduced according to their electrical threshold of activation (Nowycky et al. 1985), namely low (T-type), high (L- and P-types) and N (for **n**either L nor T-type).

The recognition of different sensitivities of the various channels to organic blocking agents and several neurotoxins led to additional differentiation. N- and P-type channels, both expressed in neurons and neuroendocrine cells, are blocked by omega-conotoxin GVIA (Hirning et al. 1988) and the funnel web spider toxin omega-Aga-IVA, respectively (Mintz et al. 1992). The L-type channels are generally expressed in neuronal and endocrine cells, in cardiac, skeletal and smooth muscle as well as in fibroblasts and kidney. Of particular interest in the context of this review are the L-type channels expressed in heart, smooth and skeletal muscle which are very sensitive to dihydropyridines (DHP, e.g. nifedipine), phenylalkalamines (PAA, e.g. verapamil), and benzothiazepines (BTZ, e.g. diltiazem). The blocking criterion led to the designation of the muscle L-type calcium channel as `dihydropyridine(DHP)-receptor' which is a misnomer as it suggests ligand-activation when in fact voltage-gating is one of the main features of the channel.

Multigene family, structure and function of voltage-gated calcium channels
Calcium channels are oligomeric proteins consisting of maximally 5 subunits: .1, .2, ., , and . (the , subunit seems only present in skeletal muscle L-type channels).

The α1 subunit. The .1 subunit shows slight variation depending on the tissue in which it is expressed. Structurally, it very much resembles the sodium channel . subunit with its four repeats (I-IV), each with six hydrophobic segments (S1-S6) thought to span the T-tubular membrane. It always contains the channel pore and several voltage sensors. The muscular L-type channel .1 subunit also contains the receptors for dihydropyridines, phenylalkalamines and benzothiazepines.

Six different .1 subunit genes (*CACNL1A1-6*) have been identified in vertebrates. *CACNL1A3*, the gene encoding the .1 subunit of skeletal muscle, has been localized to human chromosome 1q31-32 (Drouet et al. 1993; Gregg et al. 1993) and its human cDNA sequenced (Hogan et al. 1994). The mRNA was originally cloned from rabbit muscle (Tanabe et al. 1987). The gene product occurs in two isoforms, a rare 212 kD protein containing the complete amino acid sequence, and a frequent (approx. 95 %) 190 kD isoform truncated by posttranslational proteolysis at aa 1690 (DeJongh et al. 1991). The truncated form was shown to be functional (Beam et al. 1992). Additional forms seem to exist, at least in postnatal muscle (Malouf et al. 1992). S-specific mRNA sequences have been also detected in other tissues including brain and kidney (see Hofmann et al. 1994).

CACNL1A1 is located on human chromosome 12p13 (Lacerda et al. 1991; Powers et al. 1991; Schultz et al. 1993) and encodes two different splice products, i.e. the .1 subunits for cardiac (Ca) and smooth (Cb) muscle. This gene is also expressed in most excitable and many non-excitable cells such as fibroblasts. The L-type channel D encoded by *CACNL1A2* on chromosome 3p21.3-21.2 (Chin et al. 1991; Seino et al. 1992b) seems to be specific for the neuroendocrine system (brain: Williams et al. 1992; pancreatic islet: Seino et al. 1992a) as well as a splice variant of D or a channel encoded by *CACNL1A4*. Two other genes, *CACNL1A5* on chromosome 9q34 and *CACNL1A6* on 1q25-31 seem also to code for L-type .1 subunits of so far unidentified distribution (Diriong, Genomics, 1995 GDB-No.). Brain cDNAs have been sequenced and functionally expressed for various P, N, and T type channels (A, B, and E, respectively) (for review see Hofmann et al. 1994) but their human genes have not yet been localized.

The α2/δ subunit. Although the .1 subunit is able to form a calcium channel by itself, the fact that several other proteins consistently co-purify with it has led to the idea that other modifying subunits also exist. The skeletal muscle .1 subunit co-purifies with a protein of approximate molecular mass 175 kD (.2/.) that characteristically shifts to 150 kD (.2) upon reduction, with appearance of 3 peptides of 25, 22, and 17 kD (.) (Catterall 1991). The .2/. protein is produced by a single gene with its 5'-end encoding .2 and its 3'-end encoding .. The . subunit spans the membrane and anchors the extracellularly located .2 protein in the plasma membrane via disulfide bonds (Jay et al. 1991). Sequences identical or closely related to the skeletal muscle .2/. subunit have been found in a variety of tissues (.1/b), including cardiac and smooth muscle, and the nervous system (Ellis et al. 1988). The .2/. subunit from rat brain predicts an identical . protein and a splice variant of the processed .2 protein. The corresponding human gene has been localized to chromosome 7q21-22 (Iles et al. 1994; Powers et al. 1994).

The β subunit. The skeletal , subunit (,1) is a membrane protein, located intracellulary, consisting of 524 amino acids (Ruth et al. 1989). Its deduced amino acid sequence contains stretches of heptad repeat structure characteristic of cytoskeletal proteins. The corresponding human gene, *CACNLB1,* has been localized to chromosome 17q21-22 (Iles et al. 1993a). Differential splicing of the primary transcript of ,1 results in at least three isoforms: ,1a through ,1c (Ruth et al. 1989; Pragnell et al. 1991; Powers et al. 1992; Williams et al. 1992); ,1a is expressed in skeletal muscle whereas the two other isoforms are expressed in brain, heart, and spleen (Powers et al. 1992). The protein does not appear to be significantly glycosylated and is hydrophilic. Thus, it probably represents an extrinsic membrane protein and may normally be associated with the cytoplasmic aspect of the α_1 subunit. When skeletal muscle α_1 subunits were stably transfected in heterologous cells, co-expression of the skeletal muscle , subunits dramatically increased the number of DHP-binding sites but curiously had no effect on the current density (Lacerda et al. 1991; Varadi et al. 1991). However, the , subunit increased the rate of calcium current activation (Lacerda et al. 1991; Varadi et al. 1991). Similar effects have been noted for the α_1 subunit from cardiac muscle. When this protein was expressed in oocytes, rate of activation and amplitude of the calcium current were substantially increased by the co-expression of either the skeletal muscle or cardiac muscle forms of the , subunit (Perez-Reyes et al. 1992; Wei et al. 1991). Transcripts of two other genes (,1 and ,2) encoding proteins different from the skeletal muscle , subunit have been isolated from a cardiac cDNA library (Hullin et al. 1992). A fourth , subunit, ,4, has been cloned from rat brain (Castellano et al. 1993).

The γ subunit. This subunit seems to be specific for skeletal muscle. It consists of 222 aa and contains four putative transmembrane domains (Bosse et al. 1990; Jay et al. 1990). The human gene, *CACNLG*, has been localized to chromosome 17q24 (Iles et al. 1993b; Powers et al. 1993). When the , subunit was co-expressed with cardiac ,1-subunits in *Xenopus* oocytes, rate of inactivation and amplitude of the calcium currents were increased, and the voltage range of inactivation was shifted in the hyperpolarizing direction (Singer et al. 1991; Lerche et al. 1996a).

The skeletal muscle L-type calcium channel

The high density of L-type channels in the transverse tubules of adult skeletal muscle was noticed as an unusually high concentration of DHP-binding sites which made the isolation and characterization of the `DHP-receptor' possible. It is for this reason that most of our knowledge on the structure and function of voltage-gated calcium channels stems from work on this channel. Linkage of human or animal diseases with genes encoding voltage-gated calcium channel genes concerns either the ,1 subunit or the ,2/, subunit of the skeletal muscle L-type channel.

In skeletal muscle, this channel is essential for excitation-contraction coupling which, in contrast to heart and smooth muscle, does not require calcium influx from the extracellular space. The channel is involved in voltage-dependent calcium release from the sarcoplasmic reticulum, mediating contraction (for review see Melzer et al. 1995). It is characterized by a high activation threshold and slow inactivation. Its exceptionally slow activation is unique for voltage-gated ion channels.

The currents conducted by this channel have been studied mainly in frog and rat muscle both by the use of the microelectrode voltage clamp on intact fibers and the vaseline-gap clamp on cut fibers.

The channel of intact adult muscle has not yet been characterized on the single-channel level because of its location in the depth of the transverse tubular system. However, its isolation and enrichment in vesicular membranes provided the basis for the reconstitution of functional

channels in artificial lipid bilayers. Fluctuations of the current conducted by single channels were usually investigated with high concentrations of permeant divalent cations on both sides of the bilayer. Two separate conductances (9 and 20 pS when measured with symmetrical 90 mM $BaCl_2$) were found in such an isolated channel preparation (Pelzer et al. 1989). For both conductances the open probability depended on the membrane potential. The large conductance was sensitive to dihydropyridines and phenylalkylamines as well as to cAMP-dependent phosphorylation, and was therefore attributed to the L-type channel. The small conductance was not sensitive to these agents and may perhaps be related to the fast calcium current in frog muscle (Cota and Stefani 1986).

Calcium channels in developing skeletal muscle

Skeletal muscle fibers are formed either during embryogenesis by fusion of myoblasts or during regeneration by fusion of proliferated satellite cells. The early stages of this process can be followed in tissue culture by studying oligo- or multinucleated myotubes. Myotubes (or myoballs derived from them) exhibit both T-type and DHP-sensitive L-type currents (Cognard et al. 1986). Cultured human muscle cells conduct an additional high voltage-activated calcium current that resembles the N channel current as far as voltage-dependence and kinetics are concerned, but lacks N channel pharmacology (Rivet et al. 1992; Lehmann-Horn et al. 1995b; Sipos et al. 1995). In rat and mouse muscle fibers, the T-type current seems to prevail early during postnatal development, to give way later to L-type current (Beam and Knudson 1988a,b; Gonoi and Hasegawa 1988). In contrast Cognard and colleagues reported that L-type channels start first and then the T-type channels are transiently expressed (Cognard et al. 1992; 1993a,b). Experiments on fibers that had the connection of the transverse tubules to the surface membrane disrupted by glycerol treatment showed that at the later stages of development (e.g. 34 days of culture) the L-type channels are exclusively located in the T system whereas the T-type channels reside in the surface membrane (Romey et al. 1989). Both types are found in the surface membrane as early as day 9 of culture. The spatial separation apparently goes along with the appearance of well-defined triads (Romey et al. 1989).

Heterologous expression of the skeletal muscle L-type calcium channel

The mRNA for the .1 subunit of the cardiac L-type calcium channel produced functional channels when introduced into *Xenopus* oocytes (Mikami et al. 1989). On the other hand, the attempt to express cDNA of the (rabbit) skeletal muscle .1 subunit in the oocyte system did not result in functional channels. Functional expression of this cDNA was possible, however, in myotubes derived from mice with muscular dysgenesis (Tanabe et al. 1988; see below). Moreover, when this cDNA was introduced into the nuclei of the dysgenic myotubes, not only the typical slow calcium current could be recorded, but also EC coupling was restored (Tanabe et al. 1988). About 10 % of the cells surviving the injection procedure contracted either spontaneously or upon electrical stimulation. Contracting cells showed slow calcium currents when investigated with the whole-cell technique. These currents were not necessary for contraction since EC coupling remained functional after the calcium current was blocked by 0.5 mM cadmium ions. The restoration of the slow calcium current and skeletal muscle type EC coupling indicates that a protein derived from the same gene is a main constituent of both the calcium channel and the voltage-dependent mechanism that controls internal calcium release.

Hypokalemic periodic paralysis, a disease linked to *CACNL1A3*, the gene encoding the skeletal muscle L-type channel α subunit

Familial hypokalemic periodic paralysis is the most common form of the periodic paralyses in man. The condition is transmitted as an autosomal dominant trait. Its main symptom is the episodic occurrence of attacks of flaccid weakness. The attacks are associated with a decrease of the potassium concentration in the serum. They can be provoked by excessive intake of carbohydrates, strenuous exercise or by mental stress. Interestingly they do not occur during the physical stress but during a following rest, sometimes several hours later. The attacks vary in frequency, duration and severity. In severe attacks, the vital capacity may be reduced and death can occur from ventilatory failure or cardiac arrhythmia due to hypokalemia. However, usually patients show a normal life span (reviewed by Lehmann-Horn et al. 1994). Independent of the occurrence of attacks, patients develop a late onset, progressive myopathy (Links et al. 1994a). The Burmese cat may be considered as a naturally occurring animal model for the disease (Blaxter et al. 1986; Mason 1988).

The hypokalemia is assumed to be caused by stimulation of the sodium-potassium pump by insulin which is one physiological mechanism by which potassium ions are transported from the extracellular space into the intracellular compartment (Zierler and Andres 1957; Clausen and Kohn 1977; Flatman and Clausen 1979; Grob et al. 1957). Other hormones influencing the transmembrane movement of potassium are thought to contribute and thus to affect the frequency and severity of the clinical symptoms. This hypothesis is based on the observations that the onset of the disease is usually during puberty, penetrance in women is incomplete, attacks become predominant during pregnancy, and adrenaline is a specific triggering agent.

Mutations in *CACNL1A3*. A systematic genome-wide search in members of three families demonstrated that the disease is linked to chromosome 1q31-32 and cosegregates with the gene encoding the L-type calcium channel (DHP-receptor) .1 subunit (Fontaine et al. 1994) which is located in this region (Drouet et al. 1993; Gregg et al. 1993). Sequencing of cDNA derived from muscle biopsies of patients revealed so far three mutations (Figure 3). Two of these are analogous predicting arginine to histidine substitutions within the highly conserved S4 regions of repeats II and IV (Arg-528-His and Arg-1239-His, respectively), the third predicts an arginine to glycine substitution in IVS4 (Arg-1239-Gly) (Jurkat-Rott et al. 1994; Ptácek et al. 1994a; Grosson et al. 1996). The substitutions have corresponding counterparts in the . subunit of the sodium channel and those cause paramyotonia congenita by uncoupling activation from inactivation (Chahine et al. 1994). The majority of families carry either the Arg-528-His or the Arg-1239-His substitution (Elbaz et al. 1995).

Expression of cDNA of *CACNL1A3* results in functional channels only when (i) the cell system has a sarcoplasmic reticulum and triads necessary for EC-coupling and contraction and (ii) the other four subunits of the pentameric DHP receptor are co-expressed. Thus, for the study of the dysfunction of the mutant *CACLN1A3* gene product, myotubes cultured from muscle specimens of patients are the preparation of choice although they contain also normal channels. In such myotubes three voltage-dependent calcium currents can be separated (Rivet et al. 1992; Lehmann-Horn et al. 1995b; Sipos et al. 1995): (i) a rapidly activating and inactivating current with a low-voltage threshold at about -50 mV (T-type current), seen in the majority of cells, (ii) a slowly activating and inactivating current with a high voltage threshold of about -20 mV, and sensitive to dihydropyridine, and (iii) in one third of the cells, a second type of fast inward current with kinetics similar to the T-type current but with its maximum about 30 mV more positive (3rd-type according to Rivet et al. 1992).

In myotubes from patients, both arginine-to-histidine exchanges seemed to influence channel inactivation but did not alter activation (Lehmann-Horn et al. 1995b; Sipos et al. 1995). The density of the 3rd-type current was markedly increased. As this current is also seen in

normal myotubes, it is unlikely that it is conducted by mutant L-type channels. Rather, the authors suggested that calcium channel expression may be altered as a consequence of the mutation. A delay of the downregulation of the gene coding for the channel conducting the 3rd-type calcium current could account for the results.

The L-type calcium current of myotubes with the II-S4 mutation (Arg-528-His) has a normal voltage dependence, however, its inactivation curve is shifted to more negative potentials (Sipos et al. 1995) and thus resembles the altered currents seen in the presence of phenylalkylamine drugs such as gallopamil (D600) and devapamil (D888). These drugs bind to the intracellular side of segment IV-S6 of the channel (for review see Catterall and Striessnig 1992), selectively stabilizing a voltage-dependent inactive state (Erdmann and Lüttgau 1989). Since in the myotubes both the wild-type and the mutant channel is expressed, two L-type current components, a normal and a modified one, are expected. However, the presence of a monophasic inactivation curve suggested a homogenous population of channels with abnormal inactivation. This might be explained by an oligomeric arrangement of the ion-conducting channels forming a functional unit in which one mutant monomer is sufficient to produce malfunction of the entire complex. Such a functional unit might be constituted by the transverse tubular tetrads (Block et al. 1988; Lamb 1992).

In contrast to the results obtained from patient myotubes, a study of the functional consequences of the corresponding Arg-to-His exchange in the cardiac $_1$ subunit, which can be easily expressed in a heterologous cell system, did not show this shift (Lerche et al. 1996a). Since the tetradic organization of the DHP receptors is most likely lost in the cells used, the authors suggest that this arrangement is essential to create the specific functional change on inactivation observed in human myotubes.

How inactivation of the L-type calcium current is related to hypokalemia-induced attacks of muscle weakness which characterize familial hypokalemic periodic paralysis can only be speculated upon. Since electrical muscle activity is reduced or even absent during attacks (Engel et al. 1965; Links et al. 1994b), a failure of excitation is more probable than a failure of excitation-contraction coupling. Nevertheless, the hypokalemia-induced membrane depolarization observed in excised muscle fibers (Rüdel et al. 1984) might also reduce calcium release by inactivating sarcolemmal and t-tubular sodium channels, and repolarization of the membrane by activation of ATP-sensitive potassium channels restores force (Grafe et al. 1990).

The muscular dysgenesis (mdg) mouse

A mouse mutant was discovered with homozygous animals dying at birth because of non--functional respiratory muscles. The disorder is inherited as an autosomal recessive trait and was shown to be caused by a deletion of a single nucleotide in the gene encoding the L-type calcium channel (DHP receptor) $_1$ subunit (Chaudhari 1992). In the muscles of homozygous animals, the altered mRNA is present only at low levels (Tanabe et al. 1988) and $_1$ subunits are immunologically not detectable (Knudson et al. 1989). Action potentials can be elicited in these muscles but they do not trigger contractions. Mice heterozygous for the deletion are not distinguishable from normal mice.

Myotubes can be cultured from the muscle of newborns and studied in primary culture (Beam et al. 1986; Beam and Knudson 1988a,b; Adams and Beam 1989). The DHP-sensitive L-type current and excitation-contraction coupling are both absent (Beam et al. 1986). However, L-type currents were present after rabbit cDNA encoding the $_1$ subunit had been injected into nuclei of these myotubes (Tanabe et al. 1988). Ever since this detection, this expression system was successfully used for the characterization of the structure-function relationship of the L-type calcium channel $_1$ subunit.

Malignant hyperthermia

Susceptibility to malignant hyperthermia (MH) is a subclinical myopathy that is transmitted as an autosomal dominant trait. Life-threatening symptoms may occur when a susceptible individual is exposed to triggering agents such as volatile inhalation anesthetics and depolarizing muscle relaxants. MH crises are characterized by a dysregulation of free myoplasmic calcium that cause (i) increased skeletal muscle metabolism producing hypercapnia, hypoxemia, tachycardia, acidosis and elevated core temperature; and (ii) skeletal muscle symptoms such as masseter spasms, generalized muscle rigidity, increased serum creatine kinase (CK), myoglobinuria and hyperkalemia (Denborough and Lovell 1960).

In some of the affected families, the susceptibility to malignant hyperthermia is linked to the gene encoding the skeletal muscle ryanodine receptor (RyR1) (MacLennan et al. 1990; McCarthy et al. 1990), a calcium channel situated in the triadic membrane parts of the sarcoplasmic reticulum. This channel is not considered a voltage-gated channel *per se* although voltage sensitivity is generated in conjunction with the L-type calcium channels situated in the opposing triadic membrane parts of the transverse tubular system to which they are connected by the triadic `feet' structures (Francini-Armstrong 1970; for review see Francini-Armstrong and Jorgensen 1994). Several point mutations in the RyR1 gene have been detected to cause this susceptibility (MH type 1). One of the point mutations (predicting Arg-614-Cys, Gillard et al. 1991) has a counterpart in the pig genome (predicting Arg-615-Cys) which causes the porcine stress syndrome, an animal homologue of MH (Mitchell and Heffron 1982). How the mutations cause the intracellular calcium dysregulation is still a matter of debate (for review see MacLennan and Phillips 1992).

In other families with typical MH susceptibility, linkage of the condition to the RyR1 gene was excluded (Deufel et al. 1992). In one such family, linkage to chromosome 7q and the gene encoding the L-type calcium channel α_2/δ subunit was found (Iles et al. 1994). The lod score for the linkage is close to 3 and therefore establishes this gene as candidate gene for MH type 2. This gene is not yet completely sequenced.

Chloride channelopathies

Voltage-gated chloride channels in skeletal muscle

In nervous tissue, the chloride conductance is so small that it is usually neglected in considerations concerning axonal excitability. In contrast, the chloride conductance of resting adult skeletal muscle is rather large (133 $\mu S/cm^2$ in human external intercostal muscle fibers at the membrane potential of -80 mV, as compared to a potassium conductance of 42 $\mu S/cm^2$, Kwiecinski et al. 1984). Nevertheless, electrophysiological identification and characterization of the channels responsible for this high conductance turned out to be very difficult. Three different types of chloride channel were found in single-channel recordings of human myotubes (Fahlke et al. 1992), and chloride channels of intermediate (Burton et al. 1988) or large (Költgen et al. 1991) conductance were occasionally detected in membrane blebs from adult human muscle fibers, but none of these seemed to be responsible for the high conductance. After many laboratories had failed to find the major chloride channel with the patch-clamp or to reconstitute it using techniques that had turned out successful with potassium channels, the use of the vaseline-gap technique on adult rat skeletal muscle fibers finally led to the desired determination of the properties of homologously expressed major muscle chloride channel (Fahlke and Rüdel 1995). By that time the channel had also been cloned, functionally expressed and characterized

in the heterologous *Xenopus* oocyte system (Steinmeyer et al. 1991b). It had been named ClC-1, and it was shown that it is responsible for the high chloride conductance of the muscle fiber membrane. Recent immunohistochemical analysis with Clc-1 antibodies show that ClC-1 is localized at least in the sarcolemma (Gurnett et al. 1995).

Muscle diseases in man linked to *CLCN1*, the gene encoding ClC-1

As mentioned, mutations in *CLCN1* lead to several forms of myotonia, diseases characterized by a temporary muscle stiffness that can affect every skeletal muscle of the body. The first description of the classical picture of myotonia congenita was given by Asmus Julius Thomsen (1876) who had the disease himself. He already clearly described the myotonic stiffness and the non-progressive character of the disease, and correctly stated that the mode of inheritance was dominant.

In the 1950s, Becker (1957) claimed that in many families diagnosed as having myotonia congenita, the mode of inheritance was recessive, as already discussed by Thomasen (1948). In the recessive type, the symptoms were also not congenital but seemed to start at the end of the first decade of life. Becker also found that in the recessive type the myotonia was more generalized than in Thomsen's disease. Therefore he named this type `recessive generalized myotonia' and later it was considered a nosological entity that received its own entry in the standard listing of hereditary diseases (McKusick 1992). This `Beckerform' of myotonia is also characterized by a transient weakness, particularly in the arm and hand muscles, that patients experience when they want to make use of their muscles after a period of rest.

It is now clear that both the dominant and the recessive form are caused by mutations in *CLCN1* (Koch et al. 1992). The intensive search for mutations that followed this discovery showed that the dominant form is very rare, as less than 10 different families have been identified at the molecular level up to date. The recessive form is much more common, and the estimation by Becker (1977) of a frequency between 1:23,000 and 1:50,000 might still hold. Males seem to predominate at a rate of 3 : 1 when the Becker-type propositi are counted. However, family studies disclose that women are affected at the same frequency though to a much lesser degree.

In 1966, DeJong (1966) reported a very benign form of myotonia congenita with dominant mode of inheritance and called it myotonia levior. The variant is characterized by mild symptoms, late onset of myotonia and absence of muscle hypertrophy. The mutation in *CLCN1* that is responsible for this benign form was recently described (Lehmann-Horn et al. 1995a).

Pathophysiology of chloride channel myotonia

The myotonic reaction can be described in electrophysiological terms as resulting from a lowered electrical threshold and an increased tendency to react to a prolonged current pulse with repetitive activity. The pharmacologist Shirley Bryant (1969) was the first to relate these abnormalities to a congenital absence or reduction of the chloride conductance of the muscle fiber. The `low chloride conductance theory of myotonia', first developed for an animal model, the myotonic goat, and later confirmed by *in vitro* studies on excised muscles from patients with both dominant and recessive myotonia (Lipicky et al. 1971; 1979; Rüdel et al. 1988; Franke et al. 1991), explains the hyperexcitability as follows (Adrian and Bryant 1974). During physiological activity potassium leaves the interior of the muscle fibers and accumulates in the lumen of the transverse tubules. In a normal fiber with a high chloride conductance, tubular potassium accumulation causes depolarization of about 0.1 mV per action potential, not enough to be of physiological importance. In myotonic fibers with reduced chloride conductance, the same potassium accumulation causes about 10 times as much depolarization. In other words,

while in normal fibers the chloride conductance tends to clamp the membrane potential to the chloride equilibrium potential near -80 mV (Bryant 1976), the lack of this shunt in myotonic fibers leads to after-depolarization and subsequent repetitive activity.

Variation of the parameters for a mathematical membrane model that included the T system showed that the mere lowering of the chloride conductance did not cause repetitive firing (DeCoursey et al. 1981). In fact, appropriate adjustment of the sodium channel activating kinetics was necessary for a correct simulation of the experimentally observed lack of accommodation. Interestingly, corresponding abnormalities in the properties of the sodium channels were noted in myotonic muscle, both from the goat (Adrian and Marshall 1976) and humans (Lipicky 1977; Franke et al. 1991; Iaizzo et al. 1991). Variation of the degree of artificial block of the chloride conductance of normal rat muscle (Bryant 1982) showed that, for myotonia to occur, the fraction of the chloride conductance may not be larger than 30 % of the total WT membrane conductance (the rest is mainly potassium conductance). Determination of this fraction in a large number of patients having either dominant or recessive myotonia congenita showed that this fraction ranged from 0 to 66 % (Franke et al. 1991). Functional gene dosage compensation on the channel protein seems to determine the sarcolemmal density of chloride channels and the reduction of other ion conductances may be secondary effects of the myotonia (Chen et al. 1996, see also below: Myotonic Mice).

This fairly coherent `low-chloride conductance' hypothesis was tested with many animal models, e.g. the myotonic goat, rats that had been fed on a diet containing blockers of the cholesterol synthesis (see Kwiecinski 1981), and muscles made myotonic *in vitro* by replacing chloride in the extracellular fluid by an impermeant anion, or by blocking the chloride channels with monocarboxylic aromatic acids, the most potent of them being 9-anthracene carbonic acid (Bryant and Morales-Aguilera 1971). Up to the era of molecular biology it was unknown, however, whether in the hereditary myotonias the muscle chloride channels were defective or altogether missing. This question was solved when *CLCN1*, the gene encoding sarcolemmal chloride channels, was discovered.

Myotonia in the goat

About 30 years after the first description of myotonia in man (Thomsen 1876), White and Plaskett (1904) described a breed of `fainting' goats raised in Tennessee. The animals tended to have attacks of extreme muscle stiffness when attempting a quick forceful motion, so that they often fell to the ground for 5-20 s with extension of the limbs and neck. Clark et al. (1939) were the first to refer to the disease as `a form of congenital myotonia in goats'. It was excised external intercostal muscle from this strain, that the American pharmacologist Shirley H. Bryant used for his famous electrophysiological studies of the membrane conductance (reviewed by Bryant 1983). These studies led him to the conclusion that the fundamental electrical abnormality of resting myotonic fibers is a reduced chloride conductance. Studies on the same muscle from the myotonic goat also first elucidated the role of the T-tubular system in generating repetitive action potentials (Adrian and Bryant 1974). Curiously, the myotonic goat did not play a role for the finding of the genetic defect causing the reduced chloride conductance. Only long after the gene encoding the muscle chloride channel was localized and cloned for mouse (Steinmeyer et al. 1991a) and man (Koch et al. 1992), was the goat mutation in the homologous gene determined (Beck et al. 1996). It causes an Ala-885-Pro substitution in the C terminal end of the chloride channel protein (Figure 4) that results in a right shift of the activation curve.

Myotonic mice

In the late seventies two spontaneous mouse mutations were detected (as reviewed by Rüdel 1990), one in the A2G strain in London (Watkins and Watts 1984), the other in the SWR/J strain in Bar Harbor/Maine (Heller et al. 1982). The behavioral abnormalities of the affected animals were very similar, and in both mutations the traits were transmitted as an autosomal recessive trait. The British scientists were struck by the observation that from days 10-12 onwards the affected animals had difficulty in righting themselves when placed supine and therefore called the mutation `ADR' for `arrested development of righting response'. The Americans observed that shaking the cage provoked sustained extension of an animal's hind limbs, and since electrical myotonia was recorded in the EMG from the stiff muscles, this strain was called `MTO', for `myotonic'. Thorough investigations of the time course of twitches and tetanic contractions and of the excitability properties of the muscle fiber membranes showed for both the MTO (Entrikin et al. 1987) and the ADR (Reininghaus et al. 1988) that the typical myotonic features were present even in the isolated muscles. As far as the phenotype is concerned, the two models of myotonia are virtually indistinguishable (Reininghaus et al. 1988; Költgen et al. 1989). Transplantation experiments with ADR and wild type grafts showed that the ADR phenotype is based on an intrinsic muscle property (Füchtbauer et al. 1988) and electrophysiology proved for MTO (Bryant et al. 1987) and ADR (Mehrke et al. 1988) that, as in the myotonic goat, the reason for the abnormal excitability is a reduced chloride conductance. Intercrosses of the type A2G *adr*/+ x SWR/J *mto*/+ lead to the diseased phenotype indicating that *adr* and *mto* are allelic (Jockusch and Bertram 1986). The latter allele was then renamed adr^{mto}. Three further mutations in the adr allelomorphic series have been reported (Adkison et al. 1989; Neumann and Weber 1989), and the *mto* allele was mapped by conventional markers to chromosome 6 (Davisson et al. 1989). The assumption of conservation of the genomic structure in the vicinity of the *Hox 1.1* gene led Jockusch (1990) to predict that the Becker myotonia gene is located on the human chromosome 7 before the major chloride channel, Clc-1, of mammalian skeletal muscle was cloned.

When this was finally accomplished (Steinmeyer et al. 1991a; see below), it was soon shown that the spontaneous mutation of the ADR mouse consists in the insertion of a retroposon of the ETn family in the gene coding for Clc-1. This destroys the gene's coding potential for several membrane spanning domains (Steinmeyer et al. 1991b). From this and the lack of recombination between the *Clc-1* gene and the *adr* locus, it was concluded that a lack of functional chloride channels is the primary cause of mouse myotonia. Meanwhile, it was found that the adr^{mto} allele has a stop codon introduced close to the N terminus, and that the ethylnitrosourea-induced mutation adr^{K} causes the substitution Ile-553-Thr (Gronemeier et al. 1994).

Tocainide, an antiarrhythmic drug often used in human patients for the temporary abolition of myotonia (Rüdel et al. 1980) was administered to the animals by incorporating it in food pellets. It readily prevented the development of myotonia in young animals and suppressed an existing myotonia in older animals (Jokusch et al. 1988). At the same time as myotonia was suppressed the abnormalities in proportion of fiber types, myosin light chain phosphorylation and parvalbumin content were reversed. Thus, it is obvious that the biochemical abnormalities were just secondary effects of the hyperactivity. Neuromuscular transmission was found unimpaired both in ADR and MTO mice (Költgen et al. 1991).

Myotonic mouse models, mostly the ADR mouse, were also used to study the role of chloride conductance in myotubes and neonatal muscle (Wischmeyer et al. 1993) and the role of innervation on the expression of the *Clc-1* gene (Klocke et al. 1994). No indication of electrical myotonia was detected in cultured ADR myotubes and it was concluded that the low chloride conductance of myotubes is not controlled by the *Clc-1* gene as the presence of *Clc-1* mRNA is

<1 % (Bardouille et al. 1996). In contrast, in the neonatal mouse, normal excitability depends on a normal dosage of functional *Clc-1* alleles. *Clc-1* expression was found to depend on the innervation by the motor nerves, and the level of *Clc-1* mRNA was higher in fast (extensor digitorum longus) than in slow (soleus) muscle. In heterozygous adult *adr*/+ mice, the macroscopic chloride conductance of the sarcolemma is not significantly different from wild type in spite of the presence of only 50% functional *Clc-1* RNA. This indicates that a regulatory mechanism acts on the protein level limiting the density of Clc-1 channels (Chen et al. 1996).

Wischmeyer et al. (1995) were successful in recording chloride and potassium currents from single channels in lipid-supplemented sarcolemmal vesicles prepared from mouse skeletal muscle. Five different chloride channels were repeatedly identified and designated Clc-a to Clc-e, Clc-b being presumably identical with a known chloride channel (Chua and Betz 1991). The function of all these channels is so far unknown, none of them seems identical with Clc-1. Interestingly, Clc-d and Clc-e, showed some properties with the macroscopic chloride conductance and were not detected in vesicles made from ADR mouse muscle. The authors speculate that the chloride channels in the vesicles are made up of subunits of various chloride channel proteins (for multimeric structure of Clc-1, see below). Comparison of heterologously expressed ClC-1 channels with native channels (Bretag 1987) has not revealed substantial functional differences. Thus, for the reconstituted channels a physiological function has yet to be found.

The *ClC* multigene family encoding voltage-gated chloride channels

ClC-1, the chloride channel of the skeletal muscle fiber membrane, belongs to a family of voltage-gated chloride channels whose members are encoded by a gene family that has no relation with any other known ion channel gene family. Thus, ClC-1 is structurally entirely different from the epithelial chloride channels affected in cystic fibrosis.

Several electric fish of the species *Torpedo* contain at high density chloride channels in the cells of electroplax, the electric organ that develops from skeletal muscle tissue (White and Miller 1979). The cDNA encoding this channel was cloned by Jentsch et al. (1990). Expressed in *Xenopus* oocytes, it gave rise to large chloride currents. The channel conducting these currents was called ClC-0. It was the very first recognized gene product of a member of the newly detected *ClC* gene family (Steinmeyer et al. 1991b).

Meanwhile at least nine different *ClC* genes (reviewed by Pusch and Jentsch 1994 and Jentsch et al. 1995) have been discovered in mammals, several of which seem to be expressed ubiquitously. *CLCN1* encodes the major chloride channel in skeletal muscle, to be extensively discussed below. *CLC-2*, having an overall identity of about 50 % with *CLCN1* codes for a swelling-activated chloride channel (Gründer et al. 1992; Thiemann et al. 1992). Related to the same branch of the gene family are the kidney-specific channel genes *ClC-K1* and *ClC-K2* (Uchida et al. 1993) and their human counterparts *hCLC-Ka* and *hClc-Kb* (Kieferle et al. 1994). They are about 80-90 % homologous to each other, but the channels are differentially distributed along the nephron. A more distant branch of the family contains *ClC-3* (Kawasaki et al. 1994; 1995) and *ClC-4* (van Slegtenhorst et al. 1994). *ClC-3* is rather ubiquitously expressed, its function is not yet known. With *CLC-4*, expression in man is highest in muscle and brain (van Slegtenhorst et al. 1994), whereas in rat it is most prominent in liver and brain (Jentsch et al. 1995). It may play a role in patients with recessive myotonia congenita (Mailänder et al. 1996).

***CLCN1*, the human gene encoding ClC-1**

Two independent lines of experiments led to the localization of *CLCN1*, the human gene encoding ClC-1. One line made use of the myotonic mouse mutant adr (for `*a*rrested *d*evelopment of *r*ighting response', Heller et al. 1982; see above). Conventional mapping techniques and the use of restriction fragment length polymorphisms (RFLPs) resulted in localization of the *adr* locus on chromosome 6, close to the locus for the T cell receptor ß (Tcrb) (Adkison et al. 1989). This made it very probable that the Becker myotonia gene is localized in the homologous region on human chromosome 7q32-ter. Subsequently, tight linkage was shown between the TCRB locus and Thomsen myotonia (Abdalla et al. 1992).

The other, more direct way to localizing HUMCLC started by expression cloning of ClC-0, the chloride channel of the electric fish, *Torpedo marmorata* (Jentsch et al. 1990). Partial cDNAs of chloride channels of rat and mouse (Steinmeyer et al. 1991b) and human (Koch et al. 1992) skeletal muscle, ClC-1, were then cloned by homology screening. The probes obtained in this way were used to show linkage to the *adr* locus and to both Becker and Thomsen myotonia, respectively (Koch et al. 1992). The gene, located at 7q35, spans at least 40 kb and contains 23 exons whose boundaries have been located (Lorenz et al. 1994). It consists of 2964 base pairs and codes for a protein of 988 amino acids. The cDNA was functionally expressed in *Xenopus* oocytes (Lorenz et al. 1994). Primer sequences for the amplification of all exons are given in Lehmann-Horn et al. 1995.

Naturally occurring mutations in *CLCN1*

Twenty nine mutations in various exons of *CLCN1* have been discovered (Figure 4 and Table 2). Six point mutations exert partly dominant effects; five of them are missense mutations such as Gly-230-Glu (George et al. 1993a), Pro-480-Leu (Steinmeyer et al. 1994), Ile-290-Met (Lehmann-Horn et al. 1995a), Gln-552-Arg (Lehmann-Horn et al. 1995a), and Gly-200-Arg (Mailänder et al. 1996); and the sixth is a nonsense mutation which causes truncation at the very end of the protein: Arg-894-Stop (George et al. 1994b). Pro-480-Leu is present in Dr. Thomsen's offspring; Gln-552-Arg was found in a family with *myotonia levior*, a term coined by deJong for a dominant myotonia variant characterized by mild symptoms, late onset of myotonia and absence of muscle hypertrophy. With the exception of Gly-230-Glu which was detected in three Canadian families, each mutation was only detected in one single family.

The other point mutations, i.e. Phe-413-Cys (Koch et al. 1992), Val-327-Ile, Arg-496-Ser (Lorenz et al. 1994), Phe-167-Leu, Arg-300-Stop, Arg-338-Gln (George et al. 1994b), Asp-136-Gly (Heine et al. 1994), Gln-74-Stop, Tyr-150-Cys, Tyr-261-Cys, and Ala-415-Val (Mailänder et al. 1996) and the 4bp (Heine et al. 1994) and 14 bp deletions (Meyer-Kleine et al. 1994b) were detected in (approximately 60) Becker-type patients. The majority of them were heterozygous for a mutation and supposed to carry a second, not yet identified mutation. Only thirteen index patients, most of them offspring of consanguineous parents, were homozygous (Koch et al. 1992; Heine et al. 1994; Meyer-Kleine et al. 1994b; Lehmann-Horn et al. 1995a; Mailänder et al. 1996) or compound heterozygous (George et al. 1994b; Lorenz et al. 1994; Mailänder et al. 1996). Ten further mutations that have been published very recently (Meyer-Kleine et al. 1995) are shown in Figure 4 and listed in Table 2.

Analysis of the *CLCN1* gene product

ClC-1 (predicted molecular weight 110 kDa) is functional when expressed in *Xenopus* oocytes (Steinmeyer et al. 1991b) or human embryonic kidney cells (Pusch et al. 1994; Fahlke et al. 1995) without any other subunits. Its properties are very similar to those of the channel protein, expressed in native muscle (Fahlke and Rüdel 1995). The channel conducts over the whole physiological voltage range, showing inward rectification in the negative potential range. It is

activated upon depolarization and deactivated with hyperpolarizing voltage steps to a non-zero steady-state level. It is selective for chloride against bromide and iodide. Its single-channel conductance, estimated from noise analysis is very low, near 1 pS (Pusch et al. 1994). The large macroscopic chloride conductance, therefore, must result from a high channel density in the membrane. As already known from macroscopic experiments (Bryant and Morales-Aguilera 1971; Palade and Barchi 1977) the channel can be blocked by external I^- and by low concentrations of 9-anthracene carboxylic acid (Steinmeyer et al. 1991b; Pusch et al. 1994).

When the Gly-230-Glu substitution of the Canadian family (George et al. 1993a) or the Pro-480-Leu substitution of the Thomsen family (Steinmeyer et al. 1994) were inserted in the oocytes instead of wild type ClC-1, the chloride currents were completely missing (Lorenz et al. 1994; Steinmeyer et al. 1994).

Any mutation may cause either a gain of function or a loss of function. Gene dosage effects of loss-of-function mutations may lead to a recessive or dominant phenotype, depending on whether 50 % of the gene product (supplied by the normal allele) is or is not sufficient for normal function. The effect of a particular mutation on the inheritance pattern depends on the ability of mutant ClC-1 to interact with other monomers and change the function of the channel complex. Mutant ClC-1 unable to polymerize, e.g. severely truncated proteins, allow normal ClC-1 monomers (expressed by the other allele) to form normal complexes, although reduced in number (50 %). If there are no compensatory mechanisms effective, clinically normal heterozygous carriers of such mutations would have 50 % muscle chloride conductance; effects of such mutations would be recessive. Interestingly, the mutation predicting Arg-894-Stop was found to be transmitted either as dominant or recessive trait `probably depending on the genetic background' (Meyer-Kleine et al. 1995).

The number of ClC-1 monomers that make up the channel is still a matter of dispute. In experiments co-expressing wild-type and the two disease-producing mutants Gly-230-Glu and Pro-480-Leu, Steinmeyer et al. (1994) found evidence suggesting the channel to be a tetramer. Middleton et al. (1994), purifying native *Torpedo* chloride channels (ClC-0), found sedimentation properties of a dimeric protein.

In contrast to severely truncated proteins, mutant ClC-1 may be able to interact with normal ClC-1 and thus destroy or change the function of the complex. If all monomers need to be mutants for an effect, mutation of one allele leaves the majority of the complexes functional (75 % with dimers, 94 % with tetramers) and exerts recessive effects. If one mutant monomer is sufficient for an effect, only a minority of complexes will be functional (25 % with dimers, 6 % with tetramers). Such mutations exert dominant effects unless the mutant complexes function partially (Lehmann-Horn and Rüdel 1995).

Experiments with myotonia-generating drugs (Kwiecinski 1981) have shown that blockade of 50 % of the physiological chloride current is not sufficient to produce myotonic activity. This then explains the existence of recessive transmission in the case of mutations that completely destroy the gene's coding functions, such as the two myotonia-generating deletions reported above. Dominant inheritance would be explained by a mutant gene product that can bind to another protein and, in doing so, changes its function.

Structure-function relationship of ClC-1

Our knowledge of this relationship is still very limited compared to that of voltage-gated cation channels, and relevant experimental findings have only recently begun to emerge. Based on a hydrophobicity analysis of ClC-0, Jentsch et al. (1990) originally proposed 12 to 13 transmembrane spanning segments (D1-D13). The lack of a signal sequence, combined with mutational results of ClC-2, another family member, suggests a cytoplasmic location of both the

N- and the C-terminals as well as segment D13 (Gründer et al. 1992). The linker region between D8 and D9 is most probably extracellularly located, because it contains a potential site for N-linked glycosylation, which has been demonstrated to be, indeed, glycosylated (Kieferle et al. 1994; Middleton et al. 1994). Accordingly, an odd number of transmembrane spanning segments between this linker region and both the N-terminus and D13 had to be assumed and a revision of the original model was necessary. Two new models have been proposed one of them placing segment D2 outside the membrane and D10 in the cytoplasm (Middleton et al. 1994), the other placing D4 outside and leaving the topology of region D9-D12 unanswered (Pusch and Jentsch 1994). In the latter work, it was also claimed that a lysine residue in segment D12 is located at the cytoplasmic end of the channel pore.

The currents through ClC-1 channels expressed in HEK 293 cells recorded at varied extracellular and intracellular chloride concentrations and pH values were used to develop a model for ClC-1 gating (Fahlke et al. 1996). A set of two identical residues carrying a single negative charge, are postulated to function as voltage sensor. Each of the three possible conformations of this voltage sensor determines another affinity of the intracellular mouth of the pore for a gating particle that mediates the transitions between the open and closed channel. As a consequence of the three different configurations of this set of voltage sensors, current activation as well as deactivation is composed of three different components, with fast, slow and time-independent time course. The rearrangements that the two voltage sensors undergo both upon hyperpolarizing and depolarizing voltage steps, are very rapid. This model is able to explain all macroscopic gating properties described so far.

The missense mutation (Asp-136-Gly) leading to recessive myotonia congenita (Heine et al. 1994) considerably affects voltage-dependent gating without altering permeation properties. This led to the suggestion that the aspartic acid in position 136 is the voltage sensor (Fahlke et al. 1995).

The channel can be phosphorylated by activation of protein kinase C and the site of phosphorylation was postulated to be located at the vestibule of the cytoplasmic face of the pore (Rosenbohm et al. 1996).

Do other chloride channels influence the degree of myotonia?

Heterozygous carriers of Becker mutations do not display any clinical symptoms of myotonia. However, heterozygous males often exhibit myotonic discharges in the electromyogram while heterozygous females usually do not (Mailänder et al. 1996). The cause of this apparent sex predominance of subclinical myotonic signs is unclear. Sex hormones or the product of another muscle chloride channel gene, *ClC-4* (Kawasaki et al. 1994; 1995; ist doch Literatur zu Clc-3!?), encoded on the X-chromosome, could contribute to this preference. Although probably not of importance in normal muscle fibers, i.e. in the presence of abundant ClC-1 channels, chloride channels encoded by *ClC-4* expressed on both X-chromosomes could have a compensatory effect when the number of functional ClC-1 channels is reduced. A copy of *ClC-4* on the Y-chromosome has been excluded (van Slegtenhorst et al. 1994) and its location on distal Xp may allow it to escape X-chromosomal inactivation (Disteche 1995).

The importance of muscular chloride channels other than ClC-1 was also suggested by results obtained with the missense mutation Asp-136-Gly (Heine et al. 1994) expressed in human embryonic kidney cells. This recessive mutation is associated with more severe myotonia than usually presented by Becker patients. The mutant differs from the wild type ClC-1 in the gating properties causing pronounced inward rectification that permits chloride influx but prevents chloride efflux. Thus, electrical activity would cause intracellular chloride depletion and a shift of the chloride equilibrium potential to very negative potentials. With this

depletion, any additional chloride conductance that is operant in myotonia patients having mutations that do not cause this pronounced inward rectification becomes ineffective. Thus, the finding that myotonia is clinically so severe in Asp-136-Gly patients was taken as suggestive that in human skeletal muscle chloride channels other than ClC-1 (e.g. as described by Chua and Betz 1991) may be effective (Fahlke et al. 1995).

Conclusion

In addition to the clarification of the pathology of a whole group of hereditary diseases, the study of the consequences of these mutations at the levels of the whole system (patient), organ and cells (excised muscle specimens), and of the channel proteins has taught us that our current opinions on channel structure-function relations are far from being comprehensive. For instance it had been assumed that as in the potassium channel, also in the sodium and calcium channel proteins, the S4 unit is mainly responsible for channel activation. This notion has to be corrected as mutations in S4 of repeat IV were found to affect mainly channel inactivation. Moreover mutations affecting other channel domains, such as interlinkers or other intramembraneous subunits cause virtually the same alterations, not only when tested with the limited probe of the patch clamp but also on the level of the patient. The lack of mutations in other parts of the genes, in particular those coding for sodium or calcium channels, may indicate that proper function of the corresponding protein domains is essential for life. Thus the knowledge derived from the experiments of Nature, as these diseases may be looked upon by the cell biologist, provides a valuable addition to the results from site-directed mutagenesis. For a final understanding of the pathology of the diseases, e.g. triggering effects of cold or potassium, it seems that the regression from the proteins back to the cellular or even systemic levels is unavoidable.

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Figure Legends

Figure 1. Substitutions predicted in the . subunit of the voltage-gated sodium channel of skeletal muscle (transmembrane segments indicated by cylinders). Abbreviations used: HYPERPP - hyperkalemic periodic paralysis; PAM - potassium-aggravated myotonia; PC - paramyotonia congenita; HORSE - position of the mutation causing hyperkalemic periodic paralysis in Quarter horses. Conventional 1-letter abbreviations are used for wild-type amino acids and substitutions (indicated by arrows). Mutations discovered by many laboratories in 104 families.

Figure 2. Substitutions predicted in potassium channel Kv1.1 causing episodic ataxia with myokymia.

Figure 3. Substitutions predicted in the . subunit of the L-type calcium channel of skeletal muscle causing hypokalemic periodic paralysis (HYPOPP).

Figure 4. Substitutions predicted in the voltage-gated chloride channel protein of skeletal muscle (modified after Middleton et al. 1994). Abbreviations used: adr - positions of mutations in adr myotonic mouse; DMC - dominant myotonia congenita; RMC - recessive myotonia congenita.

Table 1. Mutations of *SCN4A*, the gene encoding the α subunit of the human skeletal muscle sodium channel

Genotype	Channel domain	Substitution	Exon no.	Phenotype	First report
Hyperkalemic periodic paralysis					
C2188T	$IIS5_i$	Thr-704-Met	13	permanent weakness (non)-myotonic most frequent	Ptácek et al. 1991a
G2341A	IIS6	Val-781-Ile	13	cardiomyopathy?	Baquero et al. 1995
G3466A	$(IIIS4/5)_i$	Ala-1156-Thr	19	reduced penetrance	McClatchey et al. 1992b
A4078G	IVS1	Met-1360-Val	23	reduced penetrance	Lehmann-Horn et al. 1993
A4774G	$IVS6_i$	Met-1592-Val	24	myotonic, frequent	Rojas et al. 1991
Paramyotonia congenita					
G3877A	$IIIS6_i$	Val-1293-Ile	21		Koch et al. 1995
C3938T	$(III/IV)_i$	Thr-1313-Met	22	frequent	McClatchey et al. 1992c
T4298G	IVS3	Leu-1433-Arg	24		Ptácek et al. 1993
C4342T	IVS4	Arg-1448-Cys	24	potential atrophy	Ptácek et al. 1992a
G4343A	IVS4	Arg-1448-His	24		Ptácek et al. 1992a
G4343C	IVS4	Arg-1448-Pro	24	potential atrophy	Lerche et al. 1996b
G4372T	IVS4	Val-1458-Phe	24		Heine et al. 1996

T4418C	$IVS4/5_i$	Phe-1473-Ser	24		Heine et al. 1996

Table 1. Ctd.

Potassium-aggravated myotonias					
C2411T	$IIS6_i$	Ser-804-Phe	14	overlap myotonia fluctuans	McClatchey et al. 1992b Ricker et al. 1994
A3478G	$(III/IV)_i$	Ile-1160-Val	19	acetazolamide-responsive	Ptácek et al. 1994b
G3917A G3917C G3917T	$(III/IV)_i$ $(III/IV)_i$ $(III/IV)_i$	Gly-1306-Glu Gly-1306-Ala Gly-1306-Val	22 22 22	myotonia permanens myotonia fluctuans overlap myotonia	Lerche et al. 1993 Lerche et al. 1993 McClatchey et al. 1992c Lerche et al. 1993
G4765A	$IVS6_i$	Val-1589-Met	24	myotonia	Heine et al. 1993

Table 2. Mutations of *CLCN1*, the gene encoding the chloride channel of human adult skeletal muscle

Genotype	Channel domain	Substitution	Exon no.	Mode of inheritance	Genetic status of propositus	First report
C220T	N-term.	Gln-74-Stop	2	recessive	homozygous	Mailänder et al. 1996
C313T	N-term.	Arg-105-Cys	3	recessive	heterozygous	Meyer-Kleine et al. 1995
A407G	1_e	Asp-136-Gly	3	recessive	homozygous	Heine et al. 1994
A449G	1/2	Tyr-150-Cys	4	recessive	compound heterozygous	Mailänder et al. 1996
T494G	2	Val-165-Gly	4	recessive	heterozygous	Meyer-Kleine et al. 1995
C501G	2	Phe-167-Leu	4	recessive	heterozygous	George et al. 1994b
G598A	2/3	Gly-200-Arg	5	dominant	heterozygous	Mailänder et al. 1996
G689A	3/4	Gly-230-Glu	5	dominant	heterozygous	George et al. 1993a
A782G	4/5	Tyr-261-Cys	7	recessive	compound heterozygous	Mailänder et al. 1996
C870G	5/6	IIe-290-Met	8	dominant	heterozygous	Lehmann-Horn et al. 1995a
G871A	5/6	Glu-291-Lys	8	recessive	compound heterozygous	Meyer-Kleine et al. 1995
C898T	5/6	Arg-300-Stop	8	recessive	compound heterozygous	George et al. 1994b
G950A	6	Arg-317-Gln	8	dominant	heterozygous	Meyer-Kleine et al. 1995
G979A-1	6/7	splice mutation	8	recessive	compound heterozygous	Lorenz et al. 1994
8T986C	6/7	Ile-329-Thr	9	recessive	heterozygous	Meyer-Kleine et al. 1995
G1013A	6/7	Arg-338-Gln	9	recessive	compound heterozygous	George et al. 1994b
2bp deletion	7	fs 387-Stop	10	recessive	heterozygous	Meyer-Kleine et al. 1995
T1238G	8_e	Phe-413-Cys	11	recessive	homozygous/ heterozygous	Koch et al. 1992
C1244T	8_e	Ala-415-Val	11	recessive	homozygous/ heterozygous	Mailänder et al. 1996
1262insC	8/9	fs 429-Stop	12	recessive	heterozygous	Meyer-Kleine et al. 1995
4bp deletion 1282-85	8/9	fs 433-Stop	12	recessive	homozygous	Heine et al. 1994
C1439T	9/10	Pro-480-Leu	13	dominant	heterozygous	Steinmeyer et al. 1994
14bp deletion	9/10	fs 503-Stop	13	recessive	homozygous/	Meyer-Kleine et al. 1994b

1437-50					heterozygous	
G1444A	9/10	Gly-482-Arg	13	recessive	heterozygous	Meyer-Kleine et al. 1995
A1453G	9/10	Met-485-Val	13	recessive	compound heterozygous	Meyer-Kleine et al. 1995
G1471A	10	splice mutation	13	recessive	compound heterozygous	Meyer-Kleine et al. 1995
G1488T	10/11	Arg-496-Ser	14	recessive	compound heterozygous	Lorenz et al. 1994
A1655G	12_e	Gln-552-Arg	15	dominant "levior"	heterozygous	Lehmann-Horn et al. 1995a
C2680T	C-T.	Arg-894-Stop	23	dominant	compound heterozygous	George et al. 1994b
				recessive	homozygous	Meyer-Kleine et al. 1995