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Genetic Analysis of Voltage-Dependent Calcium Channels

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Molecular cloning of calcium channel subunit genes has identified an unexpectedly large number of genes and splicing variants, and a central problem of calcium channel biology is to now understand the functional significance of this genetic complexity. While electrophyisological, pharmacological, and molecular cloning techniques are providing one level of understanding, a complete understanding will require many additional kinds of studies, including genetic studies done in intact animals. In this regard, an intriguing variety of episodic diseases have recently been identified that result from defects in calcium channel genes. A study of these diseases illustrates the kind of insights into calcium channel function that can be expected from this method of inquiry.

KEY WORDS: Voltage-dependent calcium channels; calcium release channels; mutations; disease loci; paroxsymal disorders; triplet repeats.

INTRODUCTION

Calcium channels occupy a unique position among the voltage-gated channels in that their electrogenic role is complimented by their role as a transducer of electrical signals to the biochemical domain (Hille, 1992). This is accomplished by the biochemical activity of the calcium ion which acts as a second messenger to stimulate a variety of cellular activities, including contraction, secretion, gating, motility, and transcription. Intracellular calcium concentrations are regulated by cell-membrane channels which act in concert with intracellular release channels that regulate the release of internal stores of calcium. In muscle this is thought to occur through physical interaction of the voltage-dependent calcium channel (VDCC) with the ryanodine receptor. In other cells the inositol triphosphate (IP3) receptor acts as a coincidence detector to release Ca^{2+} upon co-stimulation by Ca^{2+} and inositol 1,4,5-triphosphate (IP3).

The prototypical VDCC channel comprises multiple subunits: the α_1 pore-forming unit and several modulatory subunits, β, α_2–δ, and γ (muscle) or 95 kDa (other tissues). As reviewed elsewhere in this journal, molecular cloning of the VDCC subunit genes has identified to date six α_1 (α_{1A}, α_{1B}, α_{1C}, α_{1D}, α_{1E}, α_{1S}) genes, in addition to four β (β_1, β_2, β_3, β_4), one α_2–δ, and one γ modulatory subunit genes (Table I). These channels can be divided into two groups (L and non-L type) based on the sensitivity to dihydropiridines (DHP), a class of organic compounds that bind to and modulate the activity of L type channels. The non-L type channels, as defined by pharmacological and electrophysiological criteria, include the P, Q, N, and R types. Interestingly, the L type channel is encoded by three genes (α_{1C}, α_{1D}, α_{1S}), the P and Q types by one (α_{1A}), the N type by one (α_{1B}), and the R type by one (α_{1E}). Similar to Na^+ channel pore-forming proteins, the calcium channel α_1 subunit is a single polypeptide containing four repeats (domains I–IV) of six transmembrane segments and a pore-lining loop. Multiple splicing variants of the VDCC genes have also been identified. It has been suggested that this superabundance of genes and splicing variants implies functional specialization, and is consistent with the multitude of signaling functions required in different neural networks. A major problem in studying VDCC genes is their overlapping expression patterns. The

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Table I. Genetic Localization and Disease Association of Calcium Channel Genes[a]

Gene name	Locus symbol	Chromosomal location	Channel type	Disease association	Mutation
α_{1A}	CACNA1A	19p13.1	P/Q	MHP1	missense
				EA2	truncation
				SCA6	cag expansion
	Cacna1a	8		*tg*	missence
				tgla	truncation
				tgrmn	unknown
				tg3j	missense
α_{1B}	CACNA1B	9q34	N		
	Cchn1a	2			
α_{1C}	CACNA1C	12p13	L		
	Cchl1a1	Un			
α_{1D}	CACNA1D	3p21.3–p21.4	L		
	Cchl1a2	14			
α_{1E}	CACNA1E	1q25–31	R/T?		
	Cchr1a	1			
α_{1S}	CACNA1S	1q31–q32	L	HypoPP	missense
				MHS5	missense
	Cchl1a3	1		*mdg*	truncation
β1	CACNB1	17q21–q22			
	Cacnb1	11		KO	null
β2	CACNB2	Un			
	Cacnb2	2			
β3	CACNB3	12q13			
	Cacnb3	15			
β4	CACNB4	Un			
	Cacnb4	2		*lh*	truncation
α_2-δ	CACNA2	7q21–q22		MHS3?	
	Cchl2a	5			
γ	CACNG	17q24			
	—	Un			
Ryr1	RYR1	19q13.1	Release	MHS1	missense
				CCD	missense
	Ryr1	7		KO	null
Ryr2	RYR2	1q42.1–q43			
	Ryr2	Un			
Ryr3	RYR3	15q24–q25			
	Ryr3	2		KO	null
Itpr1	ITPR1	3p26-p25	Release		
	Itpr1	6		KO	null
				opt	
Itpr2	ITPR2	12p11			
	Itpr2	Un			
Itpr3	ITPR3	6p21			
	Itpr3	17			

[a] Mouse locus names and mutations are written in italics, human locus names and mutations are in roman. Un is unknown, KO is knockout.

clearest characterization of a given gene or splice variant may therefore come from heterologous expression systems. This strategy is limited, however, because these expression systems do not recapitulate all aspects of the differentiated muscle cell or neuron. The genetic analysis of calcium channels brings a complementary set of tools to bear on this fundamental question, at the level of the intact animal. Thus, the experimental manipulation of channel expression *in vivo* allows investigation at a level not accessible to *in vitro* preparations.

The essence of the genetic approach is analysis of the correlation between the *phenotype* and the *genotype* of the organism. This analysis embraces several

methodologies. First, in cases where specific genes are unidentified, study of phenotype inheritance, by linkage analysis, leads to discovery of genetic loci that contribute to an organism's behavior. Second, in the case of known genes, direct genetic manipulations can be used for *in vivo* experimentation. Third, in the case of discordance between genotype status and phenotypic expression, i.e., "reduced penetrance," studies can be undertaken to identify additional genetic loci which interact with the gene of interest.

Currently, genetic analysis of calcium channels means principally disease association. This has been accomplished by a variety of methods. The familiar strategy involves delimiting a disease interval and evaluating the genes shown to be contained in the interval. These can include either known, previously localized genes or transcription units found by molecular analysis of the interval. In some cases, candidate genes can be suggested by pathophysiological rationale, without linkage information. A novel method endorses the search for particular molecular lesions in affected individuals. Examples of all of these strategies will be discussed in this review.

Experimental manipulations of Ca^{2+} channels in mammals has currently progressed only to the point of construction of null alleles. More sophisticated manipulations will soon include conditional knockouts, knockin modifications, and development of multiallelic series by noncomplementation screens. *Caenorhabditis elegans* will also be discussed, as it demonstrates the feasibility and utility of screening for suppressors or enhancers, as well as saturation mutagenesis of a locus. Thus, this review, while focused on the mammalian VDCCs and release channels, will also mention results obtained in other model organisms well suited for genetic research.

DISEASE ASSOCIATIONS

Peripheral Disease

Voltage-Dependent Calcium Channels

Ironically, the first identification of a mutation in a VDCC gene was accomplished in the absence of any genetic linkage information. The original association was made in the course of developmental and electrophysiologic analyses of the muscular dysgenesis (*mdg*) mouse strain, which harbors a perinatally lethal recessive mutation that causes aberrant differentiation of skeletal muscle early in embryogenesis (Green, 1990). Initial suggestions that *mdg* might result from a defect in a VDCC gene included observations of decreased dihydropyridine (DHP) binding (Pincon-Raymond *et al.*, 1985) and lack of a slowly-activating calcium current (Beam *et al.*, 1986) in *mdg* myoblasts. The specific defect of excitation–contraction (EC) coupling was consistent with the proposed role of the DHP receptor as the agent which coupled depolarization and contraction. The then-recently cloned DHP receptor cDNA (Tanabe *et al.*, 1987), isolated from muscle, was injected into *mdg* myoblasts, restoring slow Ca^{2+} currents and EC coupling (Tanabe *et al.*, 1988). The clone was also used to probe northern blots of *mdg* RNA and detected decreased expression of DHP receptor RNA. These experiments established that the DHP receptor cDNA encoded a functional muscle calcium channel, now termed the α_{1S} channel, and that its expression was affected by the *mdg* mutation. The clone was subsequently used to isolate the *mdg* DHP receptor gene, in which a single-nucleotide deletion was identified. This lesion resulted in frameshift and premature translation termination, explaining the lack of a functional α_{1S} channel in *mdg* muscle (Chaudhari, 1992). Soon thereafter the cDNA was genetically mapped to the *mdg* locus on mouse chromosome 1 (Chin *et al.*, 1992).

Mutations in the human α_{1S} gene have recently been shown to cause hypokalemic periodic paralysis (HypoPP, OMIM #170400) (Jurkat-Rott *et al.*, 1994; Ptacek *et al.*, 1994). This disease is characterized by episodes of muscle weakness accompanied by low serum potassium levels. The attacks can be precipitated by insulin or glucose and can be averted by exercise or potassium administration. The disease shows autosomal dominant inheritance with low penetrance in women and often progresses to permanent weakness due to vacuolar myopathy with advancing age (50+) (Links *et al.*, 1994). Altered electrophysiological properties of affected muscle tissue, especially in low-potassium medium, indicated a defect in excitability (Rudel *et al.*, 1984). The genetic localization of HypoPP to chromosome 1q31–32 immediately suggested the α_{1S} gene as a candidate, as it had previously been mapped to the same interval (Fontaine *et al.*, 1994). Three causative missense mutations were quickly identified in several pedigrees. One occurs at Arg528, while the other two occur at Arg1239 (Fig. 1). These mutations are located in transmembrane segments thought to act as voltage sensors.

The location of these mutations in voltage-sensor domains suggested an effect on channel kinetics. Cultured biopsy samples from patients harboring the

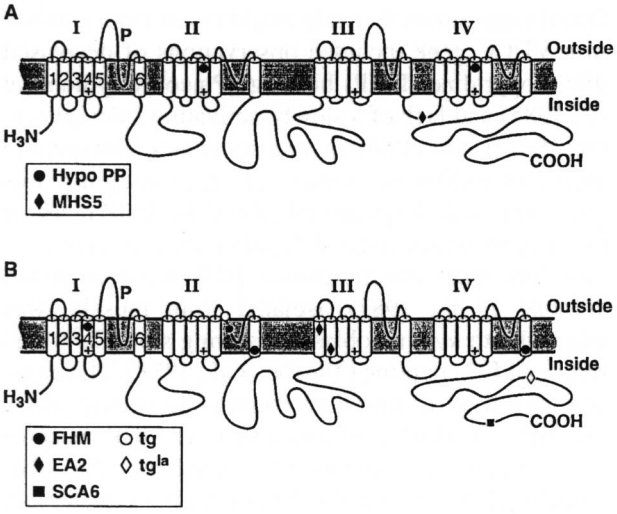

Fig. 1. Topology of the calcium channel and position of mutations. (A) The α_{1S} skeletal muscle calcium channel is mutated in hypokalemic periodic paralysis and malignant hyperthermia type 5. The positions of mutations are indicated in the figure and keyed as indicated in the legend. (B) The α_{1A} neuronal calcium channel is mutated in familial hemiplegic migraine, episodic ataxia type 2, spinocerebellar ataxia type 6, and the mouse strains tottering and leaner. The mutations are indicated in the figure and keyed as indicated in the legend. Note that the episodic ataxia mutations truncate the protein at the indicated positions, and the spinocerebellar ataxia mutation is a CAG repeat expansion in coding sequence in one RNA isoform.

Arg1239His mutation show that DHP-sensitive Ca2+ currents are reduced to 30% of normal (Lehmann-Horn *et al.*, 1995). Because the patients were heterozygous for the mutation, a proposed explanation of the 70% reduction was a dominant negative effect of the mutant channel in tetrameric complexes. Similar experiments with the Arg528 mutant myotubes indicated a monophasic −40 mV shift in the inactivation dependence of the slow current (Sipos *et al.*, 1995). However, expression of mutant cDNA (Arg528His) in L-cells showed robust channel activity with no significant alterations of channel kinetics (Lapie *et al.*, 1996) except apparently a reduced current density (reviewed in Lapie *et al.*, 1997). These results are not in agreement with the myotube data. The discordant results were explained by suggesting that some important aspect of muscle environment was not faithfully recapitulated in the L-cell system. Thus, neither the effect of the molecular lesion on the myotube channel activity, the pathophysiology of low serum K^+, nor the origin of muscle weakness is yet understood.

The mutations are clinically indistinguishable except that the Arg528His mutation has been associated with low penetrance in females (Elbaz *et al.*, 1995). Recently, slightly lowered penetrance in males has been observed in a Danish pedigree (Sillen *et al.*, 1997). Decreased penetrance could be due to modulation by steroid hormone, or perhaps the compensatory effect of another gene allele, such as a β subunit variant (given that the β subunit modulates channel activity). Likewise, mutations in other VDCC subunits may mimic this phenotype, and, in fact, additional genes are known to be involved in this disease because HypoPP pedigrees unlinked to the *CACNA1S* gene have been identified (Plassart *et al.*, 1994).

Finally, a mutation in the α_{1S} gene has been found in patients with malignant hyperthermia susceptibility type 5 (MHS5, OMIM #601887) (Monnier *et al.*, 1997). This disorder is characterized by severe hyperthermia after exposure to volatile anesthetics or depolarizing muscle relaxants and is the leading cause of death due to anesthesia. Although mutations in the ryanodine receptor (RYR1, see below) had previously been shown to cause MHS1, identification of three additional MHS loci in other kindreds implied genetic heterogeneity not evident at the clinical level. Recent efforts to resolve this issue included a genome wide scan for MHS loci, resulting in the identification of two additional MHS loci (Robinson *et al.*, 1997). The identification of a missense mutation (Arg1086His) in the α_{1S} gene unequivocally demonstrates that MHS can arise from multiple genetic lesions (Monnier *et al.*, 1997). This alteration is located in the cytoplasmic loop between domains III and IV (Fig. 1). While this does not suggest an obvious disease mechanism, Monnier *et al.* note that this region of the voltage-dependent sodium channel is mutated in paramyotonia congenita and myotonia fluctuans and that the VDCC III–IV loop has also been implicated in Ryr1 binding.

The β_1 subunit, another component of the skeletal muscle VDCC, has been investigated by gene targeting in the mouse (Gregg *et al.*, 1996). Electrophysiological studies have shown a role for modulation of channel activity by the β subunits, which are known to bind to the cytoplasmic loop between domains I and II of the α_1 subunits. Similar to the *CACNA1S*-null *mdg* strain, this mouse has disorganized striated muscle, lacks EC coupling and L-type currents, and dies perinatally. The phenotype is explained by the surprising fact that the lack of the β_1 subunit results in altered expression of the α_{1S} protein, which is not detectable by immunocytochemistry. Thus, the lack of the β_1 subunit had a much more significant impact than would be predicted from its role as a channel modulator.

In contrast, the *mdg* mouse has normal amounts and distribution of both β_1 subunit and Ryr1 protein, which show some evidence of normal targeting to the T-tubule/SR coupling junctions. Apparently, the β_1 subunit is required to stabilize the VDCC–Ryr complex.

The only other subunit of the VDCC implicated in a disease of skeletal muscle is the α_2–δ subunit. Although no definitive mutations have been demonstrated, linkage of this gene to a MHS locus segregating in one pedigree indicates that it is a candidate for this disorder (Iles *et al.*, 1994).

Calcium Release Channels

The ryanodine receptor is intimately associated with the muscle VDCC and is the second component of the excitation–contraction apparatus. The receptor's name derives from strong binding of the plant alkaloid, ryanodine, which acts as a channel agonist. This channel is a homotetramer of 565,000 Da subunits and functions to release Ca^{2+} from the sarcoplasmic reticulum after depolarization of the transverse tubules. In skeletal muscle this channel is gated mechanically by the T-tubule voltage sensor (a.k.a. α_{1S}), as well as being ligand-gated by Ca^{2+}, adenosine, and caffeine. Thus, this is referred to as Ca^{2+}-induced Ca^{2+} release current. The protein has transmembrane segments and a large cytoplasmic "foot" that forms part of the triad junction associated with tetrad arrays of α_{1S}. There are three genes: *RYR1*, predominately expressed in skeletal muscle; *RYR2*, predominately expressed in heart muscle; and *RYR3*, expressed in brain and muscle.

The Ryr1 Ca^{2+} release channel has been implicated in two human disorders: malignant hyperthermia susceptibility type one (MHS1, OMIM #145600) and central core disease (CCD, OMIM #117000). These muscle disorders are closely associated at the clinical level. MH episodes are observed after exposure to triggering agents which include certain volatile anesthetics and depolarizing muscle relaxants (Mickelson and Louis, 1996). Muscle rigidity and hyperthermia are the direct result of aberrantly increased Ca^{2+} concentration in skeletal muscle. Clinical diagnosis is accomplished by *in vitro* analysis of the contracture response of a muscle biopsy sample to caffeine and halothane (reviewed in Rosenberg and Fletcher, 1994). CCD is an early-onset myopathy whose notable feature is proximal muscle weakness. Diagnosis of CCD requires histological analysis of muscle due to the extreme clinical variability of CCD, with symptoms ranging from none (normal muscle strength) to severe muscle weakness (Shuaib *et al.*, 1987).

Physiological investigations identified defects in Ca^{2+}-stimulated Ca^{2+} release in samples from an MH patient (Endo *et al.*, 1983). Subsequently, the Ca^{2+} release channel was cloned (Takeshima *et al.*, 1989; Zorzato *et al.*, 1990) and mapped to 19q13.1 (MacKenzie *et al.*, 1990) where an MHS locus had been localized (McCarthy *et al.*, 1990). Analysis of an MHS pedigree indicated tight linkage of MH and *RYR1*(-MacLennan *et al.*, 1990). Mutations in *RYR1* have been identified in about 20% of MHS families (MacLennan and Phillips, 1995). The description of the complete gene structure (104 exons spanning 200,000 nucleotides) should facilitate mutation hunting (Rouquier *et al.*, 1993). The known mutations, all missense substitutions, cluster in two locations, both in the foot region of the protein (reviewed in MacLennan and Phillips, 1995). The molecular basis for the aberrant Ca^{2+} homeostasis has not been determined unequivocally, in part because of the difficulty in obtaining experimental material. Genetic heterogeneity, due not only to the fact that the patients are heterozygotes, but also because patients may actually be carrying mutations in genes other than *RYR1*, further complicates the analysis. Analyses of transfected mutant cDNAs, however, have consistently indicated an increased sensitivity to activating ligands and/or reduced sensitivity to inhibitors (Otsu *et al.*, 1994; Richter *et al.*, 1997; Treves *et al.*, 1994).

A homologous disorder, porcine stress syndrome, occurs with recessive inheritance in swine and is caused by a single mutation (Arg615Cys) that is found in all domestic breeds of lean pigs (Fujii *et al.*, 1991). The identical mutation accounts for about 5% of human MHS1 cases. Aside from the different mode of inheritance, episodes in swine can be induced by stress (in addition to pharmacological agents), which is rarely, if ever, the case in humans. In addition, the chronic effect is muscle hypertrophy, a desirable phenotypic feature which was originally selected for in the breeds. The ready availability of a homozygous, single allele preparation has resulted in a far more comprehensive understanding of the molecular effect of the Arg615 mutation in the pig myotube. Results indicate increased rates of Ca^{2+} release, increased open probability, increased affinity for ryanodine, and perhaps a small increase in stimulatory Ca^{2+} affinity (reviewed in Mickelson and Louis, 1996).

Once MHS-causing mutations were discovered in the *RYR1* gene (Gillard *et al.*, 1992; Gillard *et al.*,

1991) linkage of CCD to the *RYR1* locus on 19q13 was investigated and demonstrated (Kausch *et al.*, 1991; Mulley *et al.*, 1993). The supposition that these clinically dissimilar diseases were allelic was confirmed by the discovery of CCD-causing mutations in the *RYR1* gene (Quane *et al.*, 1993; Zhang *et al.*, 1993). Moreover, it has been proposed that the allelic nature of these disorders can be explained by the nature of the molecular defect. It has been suggested that CCD may result from more severe mutations that chronically overload the sarcoplasmic reticulum Ca^{2+} homeostasis machinery and damage mitochondrial function (MacLennan and Phillips, 1995; Quane *et al.*, 1993). However, the effects of these mutations on basal levels of cytosolic calcium are controversial. The variability in CCD expression and/or progression may be due to additional interacting genetic factors.

Both *Ryr1* and *Ryr3* have been ablated in the mouse by a gene-targeting strategy (Takeshima *et al.*, 1994, 1996). The homozygous deletion of *Ryr1* severely disrupts muscle differentiation (similar to *mdg*), and results in absence of EC coupling and almost complete loss of Ca^{2+}-induced Ca^{2+} current. However, residual *Ryr3* Ca^{2+} current is detectable (Takeshima *et al.*, 1995). In contrast, the loss of the Ca^{2+}-sensitive Ca^{2+} release current carried by *Ryr3* results in no detectable histological abnormalities. The sole effect appears to be that *Ryr3* mutant mice are mildly hyperactive. Double mutants (*Ryr1* and *Ryr3* null) have more severe muscular degeneration and no detectable calcium release current (Ikemoto *et al.*, 1997).

In summary, genetic lesions of various components of the skeletal muscle EC apparatus give rise to a spectrum of dominantly inherited, episodic, and clinically variable myopathies including MHS5, HypoPP, MHS1, and CCD (Table I). Only a small percentage of both the disease loci and the mutations in known disease genes have been identified to date. The identification of additional genes that underlie unlinked yet clinically similar disorders, e.g., MHS2-4, as well as modifier loci, may well identify additional components of the EC apparatus and/or pathway.

Interesting genetic features of the diseases include the fact that the human disease occurs in heterozygous patients and that none of the lesions are apparently null mutations. The phenotypes could be due to haploinsufficiency, but the HypoPP data suggests there is a dominant negative effect. This is consistent with the evidence that the muscle VDCCs and the Ryrs assemble in tetramers. An intriguing difference between man and pig is the recessive inheritance of the disease in swine. The lack of homozygous patients may be due simply to the low incidence of the allele in the population. Likewise, inbreeding of the pig may have co-selected a mitigating allele in another locus. It is clear, however, that homozygous null mutations of *Cchl1a3*, *Cacnb1*, and *Ryr1* in the mouse interfere with the normal differentiation of muscle and are perinatally lethal. Thus, the collection of mutations discovered in the clinic may simply be part of a subset that are compatible with normal development and postnatal viability. The *Ryr3* null phenotype is an example of how mutations in genes whose function can be subsumed by other components of the EC apparatus may not be detected. Thus, it is likely that the complete spectrum of relevant mutations will only be obtained in a suitable animal model by a combination of systematic mutagenesis and screening, and targeted mutation.

So far, in no case is the molecular mechanism of the disease understood. In part, this is due to the difficulty of obtaining experimental material. In addition, the analysis of HypoPP mutant cDNAs by heterologous expression systems suggests that investigations *in vitro* will be stymied by lack of complete fidelity with the muscle environment. Both these considerations further illustrate the need for a suitable animal model in which mutant alleles can be expressed *in vivo* in a controlled manner.

Central Disease

Voltage-Dependent Calcium Channels

Additional evidence that different lesions of a single calcium channel can give rise to a dissimilar clinical syndromes is provided by the α_{1A} locus. By 1995, genetic linkage studies in human had determined that both familial hemiplegic migraine (FHM, OMIM #141500) and episodic ataxia type 2 (EA2, OMIM #108500) were located on chromosome 19p13 (Joutel *et al.*, 1993; Kramer *et al.*, 1995; Teh *et al.*, 1995; von Brederlow *et al.*, 1995). FHM is distinguished by dominant inheritance of migraine with aura, ictal hemiparesis, and cerebellar atrophy in some cases. Episodic ataxia is recognized by stress or exercise-induced ataxic attacks associated with interictal nystagmus. In some cases progressive vermal cerebellar atrophy is detected. A description of the clinical heterogeneity of EA2 is provided in Baloh *et al.* (1997). The precedent of phenotypically dissimilar but allelic, dominant, acetazolamide-responsive channelopathies

prompted a search for an ion channel in the FMH/EA2 critical region. A successful positional cloning effort led to the identification of mutations in the α_{1A} gene in both diseases (Ophoff *et al.*, 1996). Four alleles of FMH have been identified as missense mutations; these are located in the IS4, II pore lining, IIS6, and IVS6 domains. Two EA2 alleles cause truncation of the protein in the IIIS1 and IIIS2 domains (Fig. 1). The IIIS1/2 truncations only encode half the protein. This unusual result, if the truncated proteins are truly inactive, suggests that EA2 may arise from haploinsufficiency. The CNS-restricted expression of this gene precludes the functional study of the mutant channel in biopsy preparations.

Contemporaneously, a positional cloning effort was underway to identify the mouse tottering (*tg*) locus. The tottering strain exhibits mild ataxia and rare tonic/clonic seizures, but no gross histological abnormality has been detected. Electroencephalograph recordings show frequent (1/min) spontaneous generalized spike and wave discharges that are accompanied by arrested movement, a hallmark of absence epilepsy (Noebels and Sidman, 1979). Genetic mapping indicated linkage to the leaner (tg^{la}) locus, a mutation which caused severe ataxia and progressive cerebellar degeneration, and these strains were shown to be allelic by breeding tests (Tsuji and Meier, 1971). High-resolution mapping localized the mutation to a region of mouse chromosome 8 which shares conserved synteny with human chromosome 19p13. In the course of investigating this interval, the mouse α_{1A} gene was discovered and subsequently found to be mutated in these strains: *tg* is a missense mutation in the second pore-lining region and tg^{la} is a truncation of the protein near the carboxy terminus (Fletcher *et al.*, 1996) (Fig. 1). The leaner mutation decreases Ca^{2+} currents, while the tottering mutation affects synaptic currents: neither are null mutations (Nancy Lorenzon, Kurt Beam, Sarah Caddick, Doug Coulter, David Hosford, Colin Fletcher, Neal Copeland, Nancy Jenkins, unpublished observations).

At the same time, yet another genetic screening strategy converged on the α_{1A} locus. This strategy exploited the recent finding that triplet repeat expansions (CAG, CTG, CGG, GAA) underlie a number of neurological disorders (Warren, 1996). Given that polyglutamine-encoding CAG repeat expansions have been implicated in a growing list of neurodegenerative disorders, the molecular search for such alterations in affected pedigrees has been adopted as a screen for mutated genes. Zhuchenko *et al.* (1997) identified expanded triplet repeats in the α_{1A} gene in eight unrelated patients suffering from progressive ataxia. Termed spinocerebellar ataxia type 6 (SCA6, OMIM #183086) by the authors, this disorder is recognized by ataxia that progresses over 20–30 years, nystagmus, and occasional cerebellar atrophy. The expansion is present near the 3′ end of the α_{1A} gene and affects coding sequence in an isoform of the mRNA which, by virtue of a five-nucleotide insertion just before the stop codon, extends the open reading frame into what had previously been considered 3′ untranslated sequence (Fig. 1). This observation has been confirmed in European and Japanese pedigrees with consistent molecular features: this expansion may account for as much as 30% of ataxia cases in Japan (Matsumura *et al.*, 1997, 1997). How this affects function in either form of the message is a bit of a mystery. The repeat size is not as large as is found in other CAG diseases (36–121), which require at least 36 repeats to be deleterious. The CAG tracts in SCA6 patients are also not unstable, unlike other triplet expansions, which tend to increase in size between generations. The size and instability of the repeats correlate with anticipation seen in these disorders, in that longer repeats accumulate between generations and are associated with earlier onset and more severe pathology. Anticipation has not been noted in SCA6, although disease severity does correlate with repeat size. In the case of SCA6, it has been proposed that the function of the channel *per se* is affected.

The implications of the tottering results were appreciated by researchers who were familiar with lethargic (*lh*), a phenotypically similar mouse strain that is also a model for absence epilepsy. Thus, an evaluation of candidate calcium channel genes that mapped near *lh* was undertaken. Lethargic was found to harbor a splicing mutation in the β_4 subunit gene that resulted in significantly decreased mRNA levels as observed by Northern blot analysis (Burgess *et al.*, 1997). Presumably, the decreased amount of β_4 subunit affects the function of the P/Q channel, given that the β_4 protein associates most strongly with the α_{1A} subunit. This observation gives further credence to the role of HVA Ca^{2+} flux as a trigger in absence epilepsy.

Calcium Release Channels

The second family of internal calcium release channels are the inositol triphosphate (IP3) receptors, which are large (313,000 Da) ligand-gated channels

that release Ca^{2+} stores from the endoplasmic reticulum (Furuichi *et al.*, 1989; Mignery *et al.*, 1989). IP3 is a second messenger, released subsequent to binding of particular neurotransmitters, hormones, and growth factors and, though gated differently, these channels can be considered analogous to the ryanodine receptors (Mikoshiba, 1993). Currently, the IP3 receptor gene family is thought to comprise five members that are expressed in a wide variety of tissues. The IP3R type 1 gene is most highly expressed in the Purkinje cells of the cerebellum. In the mouse this channel has been ablated by gene targeting (Matsumoto *et al.*, 1996). The IP3R1 null mouse suffers from ataxia, opisthotonus, and epileptic seizures, although it is free of gross histological abnormalities. In fact, Purkinje cell electrophysiology revealed no significant alterations except for a more rapid decay of parallel fiber-mediated excitatory postsynaptic current. The signature behavioral phenotype, coupled with known linkage of the IP3R1 gene to the opisthotonus (*opt*) locus, led the authors to suggest that the spontaneous *opt* mutant strain likely was allelic to the knockout strain. This was shown to be the case, as the *opt* mouse contains a genomic deletion of two exons, which results in expression of low amounts of the mutant isoform (Street *et al.*, 1997).

In summary, the functional diversity of channel-expressing tissues in the CNS is reflected in a wider spectrum of disease phenotypes, as compared to skeletal-muscle calcium-channel lesions. The curious feature of these syndromes are the distinct phenotypes observed in selected cell types. For instance, although α_{1A} is widely expressed, neuronal degeneration has been observed only in the cerebellum. Even more striking is the selective nature of the atrophy, best illustrated in leaner mice: alternating stripes of Purkinje cells degenerate. This selective sensitivity to the mutation is entirely consistent with the idea of functional specialization of cells. Additional manifestations of disease, e.g., migraine and epilepsy, indicate that other systems are not spared involvement. Although these are all late-onset disorders that do not affect development, none are obviously frank nulls. It may be that null alleles, or homozygous missense mutations in human, will not be compatible with normal development.

CHANNEL MUTATIONS IN NONMAMMALIAN SPECIES

Two nonmammalian organisms, *Drosophila melanogaster* and *Caenorhabditis elegans*, a fly and a worm, are model systems, well suited to genetic analysis, that have made profound contributions to our understanding of development in mammalian species. Many observations, resulting from application of sophisticated genetic techniques to model systems, have been successfully extrapolated to investigation of mammalian biology. The principal advantages of model organisms from the neurogenetic standpoint are rapid gestation times, the ability to generate large populations for screening, well-described fate maps and morphogenic programs, fairly simple adult structures, and yet retention of discernible behavior and learning. Moreover, the facility of mosaic analysis and cell ablation (in *C. elegans*) allows identification of the structure or cell type in which the mutation acts. Thus, these animals strike an advantageous balance between simplicity and complexity, in terms of development, structure, and function. Finally, the imminent completion of the *C. elegans* genome sequencing project will provide an invaluable resource for mutation identification. Since these systems can provide entry into the biological pathways for which calcium channels may be a central crossroads, it is useful to consider the current state of genetic characterization of VDCCs in these animals.

Drosophila melanogaster

Compared with other voltage-sensitive ion channels, the calcium channel genes are less well characterized in the fly. Electrophysiological and pharmacological analysis suggests the existence of a number of different channels types in the fly (Greenberg *et al.*, 1989; Leung *et al.*, 1989; Leung and Byerly, 1991; Pauron *et al.*, 1987). To date two α_1 subunit genes have been cloned in Drosophila (Smith *et al.*, 1996; Zheng *et al.*, 1995). These genes, Dmca1D and Dmca1A, are similar to L-type and non-L-type α_1 subunits, respectively. The Dmca1A gene was identified in a search through a genetic interval containing several mutant loci, including the courtship song mutation, *cacophony* (*cac*), the visual pathway mutation, *nightblind-A* (*nbA*), and the lethal mutation *lethal(1)L13* (Smith *et al.*, 1996). The *cac* mutation alters the number of cycles within a tone in the courtship song, involving either neuronal or muscle physiology. Nightblind alters the phototactic and optomotor behavior of affected flies. Physical mapping of four inversion or deletion breakpoints in this gene strongly implicates the Dmca1A gene in these mutant pheno-

types, although the specific molecular lesions in the *cac* and *nbA* strains have not been identified.

A homolog of the IP3 receptor has been cloned in the fly (Hasan and Rosbash, 1992) and mutation analysis indicates that it is lethal at the larval stage following delayed moulting (Venkatesh and Hasan, 1997). The immediate consequence of the IP3 receptor mutation is decreased release of the steroid hormone ecdysone, which promotes molting. In fact, exogenous ecdysone partially rescues the phenotype. Thus, neuropeptide hormone induction of ecdysone synthesis and release appears to require IP3 signaling. The effect of embryonic lethal mutations can be studied in adult flies by construction of chimeric animals. This has been done with IP3 mutants, and the role of IP3 receptor has been investigated in phototransduction, a model phospholipase C pathway (Acharya *et al.*, 1997). Interestingly, the IP3 receptor is not required in this pathway.

Caenorhabditis elegans

Genes similar to L-type and non-L-type α_1 subunits, as well as α_2–δ and internal release channels, have been identified in the worm.

The egg laying defect-19 (egl-19) gene is most homologous to α_1 subunits encoding L-type channels (Lee *et al.*, 1997). Recessive and dominant mutations at this locus were isolated because affected worms exhibit uncoordinated behavior and defects in egg-laying and pharyngeal pumping. The site of action of the gene has been localized to muscle: loss-of-function alleles show weak contraction while gain-of-function alleles show hypercontraction and delayed repolarization (Avery, 1993; Raizen and Avery, 1994). Expression studies of this gene have shown expression in some neurons in addition to muscle cells. Over 20 mutant alleles have been isolated at this locus, including null mutations that are paralyzed at early larval stages.

A ryanodine receptor homolog has also been isolated by molecular cloning (Sakube *et al.*, 1993) and identified as the unc-68 locus (Maryon *et al.*, 1996; Sakube *et al.*, 1997). Null mutants that lack ryanodine binding are viable and have normal striated muscle, but are uncoordinated. This indicates that Ryr-1, although it contributes to muscle function, appears to be dispensable for EC coupling and muscle differentiation. It has been suggested the IP3 receptor may play a significant role in nematode muscle, and that Ryr-1/VDCC coupling in vertebrate skeletal muscle is a late evolutionary specialization (Sakube *et al.*, 1997). These mutants also show altered response to ketamine. Additional screens for genes that affect sensitivity to anesthetics have identified eight loci, ordered in a three-step genetic pathway, that affect response to volatile anesthetics (Morgan *et al.*, 1990; Sedensky *et al.*, 1994; Sedensky and Meneely, 1987). Analysis of the two loci whose halothane hypersensitivity can be blocked by dantrolene may identify proteins that interact with Ryr-1.

An α_1 gene most similar to non-L-type channels has also been identified. Mutant alleles at this locus were isolated in screens for failure to adapt to exogenous serotonin and dopamine (Schafer and Kenyon, 1995). The acute response to dopamine is decreased movement and egg-laying, with recovery after several hours. Serotonin decreases movement, and initially spurs egg-laying; adaptation results in accumulation of unlaid eggs. Mutant worms show specific failure in adaptation. The mutants were found to be allelic with the previously described unc-2 locus. Unc-2 mutant worms were also found to be adaptation defective and hypersensitive to exogenous serotonin (Schafer *et al.*, 1996). Mosaic and expression analysis suggests that unc-2 acts in two neurons that innervate muscle, and may regulate the secretion of an FMRFamide-like petide that modulates response to serotonin. The mutation in the mu74 allele of unc-2 has been identified as a microdeletion of the IVS2 transmembrane domain.

The interaction of α_2–δ gene mutations (unc-36) with the unc-2 gene has also been investigated (Raymond Lee, personal communication). Double mutants are paralyzed, while each mutation independently only results in uncoordinated movement. Compound heterozygotes are defective in egg-laying, although single heterozygotes are normal. Analysis of additional alleles of both loci will allow the further dissection of α_1 and α_2–δ interactions.

In summary, genetic analysis in these organisms is greatly facilitated by the ability to screen populations for behavioral deficits and altered responses to neurotransmitters or pharmacological agents. Moreover, the construction of compound mutations allows the definition of genetic pathways. Combined with the complete genome sequence, the worm should be an excellent system to identify and analyze all the components of the VDCC and EC apparatus.

PROSPECTS

The genetic analysis of calcium channel function has identified a wide range of phenotypes associated

with mutations in voltage-sensitive and calcium release channels. To date, only a limited number of calcium channels have been identifed as disease genes and only a small percentage of the mutations have been described. Thus, the observations of the past several years represent only the initial steps of this type of analysis.

It is expected that the experimental manipulation of calcium channel function will allow more specific questions to be formed. For instance, conditional nulls can be generated that will remove a channel in specific neurons or at specific ages. More delicate deletions can be designed to remove only a particular isoform. These techniques can be expected to be more selective than pharmacological techniques (though not reversible!).

Direct approaches can be supplemented by non-complementation screens with existing alleles to isolate mutations that would not be predicted. Few of the lesions described above would have been designed. In addition, behavioral screens to isolate phenocopy mutations which occur in unlinked loci may allow the identification of interacting proteins. The ultimate success of these screens will not depend on improvements in genetic technology but rather in the development of more sophisticated phenotypic assays to uncover subtle effects on behavior. As in other fields, the results obtained in simpler organisms may guide experimentation in vertebrates.

These phenotypes described above range from easily interpreted effects on channel function to complex pathologies that reveal unexpected sensitivities to alterations in channel activity. Only in an extremely limited number of cases is the electrophysiological behavior of a mutant allele well understood. The task at hand for investigators is to describe the molecular mechanisms which cause distinct pathology in selected cell types. Nonetheless, the preliminary results described here are entirely consistent with the notion of functional specialization of calcium channels.

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