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Molecular biology of neuronal voltage-gated calcium channels

Hemin Chin

Genetics Research Branch, Division of Basic and Clinical Neuroscience Research, National Institute of Mental Health, National Institutes of Health, Bethesda, Maryland, U.S.A.

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Introduction

Calcium ions are important intracellular messengers mediating a number of neuronal functions including neurotransmitter release, neurosecretion, neuronal excitation, survival of eurons, and regulation of gene expression. The entry of calcium across the plasmamembrane in response to membrane depolarization or activation of neurotransmitter receptors represents a major pathway for regulating the intracellular level of calcium. While much less is known about the receptor-operated calcium entry mechanism, a remarkable progress has been made in our understanding of the structure, function and regulation of the voltage-gated calcium channels (VGCC). This brief review describes such recent studies on molecular biology of calcium channels, focusing on the involvement of genetic defects in calcium channel genes in neurological diseases.

Molecular diversity and differential expression of calcium channels in the nervous system.

Multiple types of VGCCs have been identified in neural tissues based upon a combination of biophysical and pharmacological criteria. They are a low voltage-activated (LVA) T-type and high voltage-activated (HVA) L-, N-, P/Q- and R-type channels. In this section, discussion is focused on the HVA calcium channels and recent cloning of cDNAs encoding a LVA T-type calcium channel will be discussed later. Extensive molecular cloning and bio-chemical efforts in recent years have further defined subunit composition, structural features and functions of each of the subunits associated with HVA calcium channels, and their gene structures (for a review; see Snutch and Reiner, 1992 ; Dunlap *et al*., 1995 ; Gainer and Chin, 1998). VGCCs are multisubunit complexes composed of the poreforming α_1 subunit and the accessory α_2-δ and β subunits (Figure 1).

The cloned α_1 subunit exhibits structural features common to voltage-gated cation channel gene families and is capable of directing expression of calcium channel activity in heterologous expression systems. In the central nervous system (CNS), VGCCs are expressed by five distinct a1 subunit genes (α_{1A}, α_{1B}, α_{1C}, α_{1D} and α_{1E}), which exhibit further variations due to alternative splicing of the primary RNA transcripts. The α_{1C} and, α_{1D} subunit genes encode dihydropyridine (DHP)-sensitive L-type channels, while the three other α_1 subunit genes (α_{1A}, α_{1B} and α_{1E}) give rise to DHP-insensitive P/Q-, N- and R-type channels, respectively. The α_2 and δ subunit proteins are produced by proteolytic cleavage of a larger precursor produced by the single α_2-δ gene (Table 1). Three alternatively spliced variants of the α_2 subunit are expressed in a tissue-specific manner. Two variants have been isolated from the brain and skeletal muscle (Kim *et al*., 1992; Williams *et al*., 1992), and a distinct third splice variant which is expressed in glial cells has been recently identified (Puro *et al*., 1996). In addition to the gene encoding the skeletal muscle β_1 subunit, three other β subunit genes (β2, β3 and β4) have been isolated thus far. Like the α_1 subunits, differential splicing of the primary transcripts from the β subunit genes generate multiple isoforms (Table 2).

The expression of cloned calcium channel by using α_1 subuit alone or in combination with α_2 and β subunits in heterologous expression systems such as Xenopus oocytes has allowed for the assessment of the roles and contributions of the accessory subunits in channel func-tion. Electrophysiological and biochemical characteristics of the expressed calcium channels are greatly influenced by the coexpressed β subunits (Sather *et al*., 1993). In fact, different combinations of the diverse α_1, α_2 and β subunit transcripts in a cell could provide a possible mechanism to generate various calcium channel properties observed in the CNS.

The transcripts encoding HVA calcium channel subtypes are differentially localized in different regions of the brain and as well as in individual neurons within the specific brain regions. While ω-contotoxin-sensitive N-type channels encoded by α_{1B} gene is widely expressed throughout adult rat brain, DHP-sensitive L-type and P/Q-type channels, encode by α_{1D} gene and α_{1A} genes respectively, exhibit distinct patterns of their mRNA expression (Figure 2). As previously noted (Chin *et al*., 1992a), the α_{1D} transcripts are predominantly expressed in the brain regions important for neuroendocrine function such as pituitary and pineal gland, and olfactory bulb and dentate gyrus of hippocampus. In contrast, the α_{1A} transcripts are most abundant in the pyramidal cells of CA3 region of hippocampus and the Purkinje and granule cells of cerebellum. However, expression patterns of VGCC subtypes at the cellular level are much more complex in

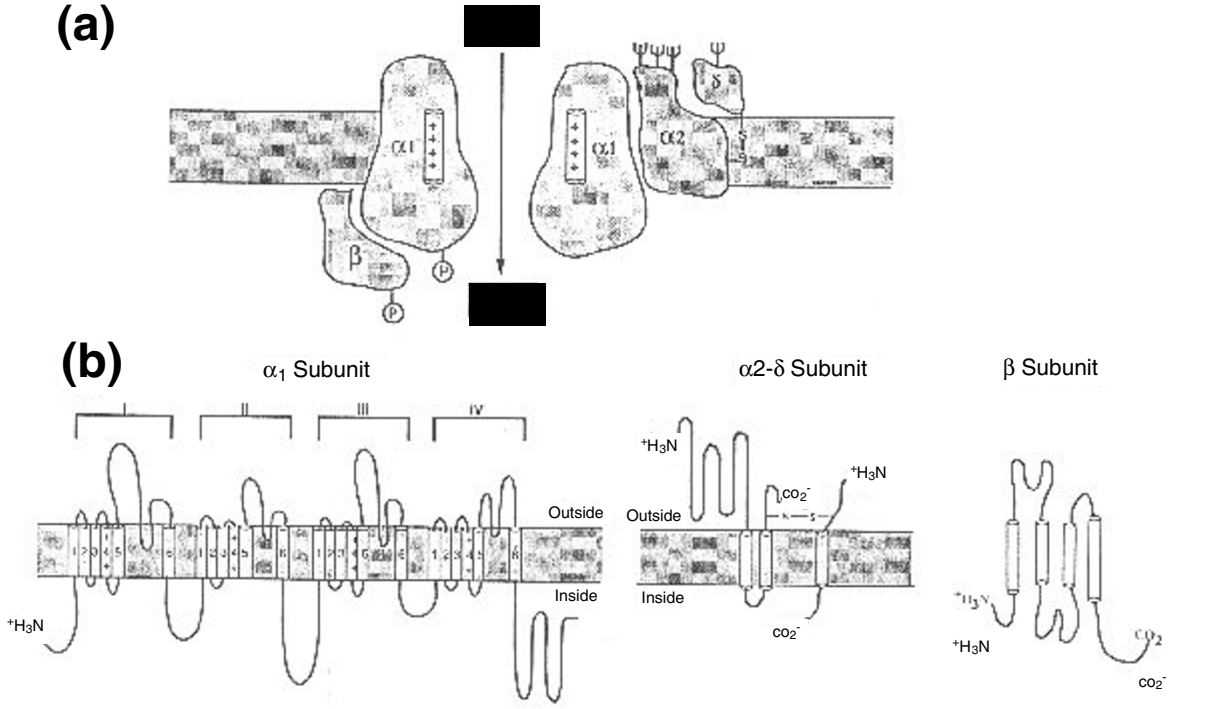

Figure 1. Calcium channel subunit structures. **(a)** The subunits of a DHP-sensitive L-type calcium channel are illustrated. The pore-forming a1 subunit contains hydrophobic =transmembrane domains. The putative voltage-sensing domain (S4) are also indicated as + charges. The α_2 and δpolypeptides are heavily glycosylated and are linked to one another by disulfide bonds (-S-S-). The b subunit proteins are cytoplasmic. The primary structure of the α_1 and β subunit cDNAs indicate that there are multiple consensus sequences for phosphorylation by various protein kinases as indicated by circled P's (adapted from Gainer and Chin, 1998). **(b)** Membrane topology of individual calcium channel subunits as determined by primary structure analysis. Cylinders represent predicted a helical segments. The α_1 subunit consists of four repeating homologous units, indicated by the Roman numerals. Each of these contains six transmembrane a helical segments. The fourth a helical segment of each repeating unit (indicated with + charges) contains a positively charged amino acid (either lysine or arginine) in every third position and is involved in voltage-sensing. The α_2 and δ polypeptides are products of a single gene and are post-translationally cleaved and linked by a disulfide bond. The cytoplasmic β subunit is peripherally associated with the a1 subunit as shown in a.

Table 1 The Pore-Forming α1 Subunit Genes and Their Splice Variants. Data adapted from Dunlap *et al.*,1995. Abbreviations: HVA,MVA or LVA are high, medium, or low voltage activated current, respctively.

Gene	Electrophysiology	Phamacological Blocker	Splic Variants	Location	GenBank Accession No/References
α1A	HVA,P-or Q-type	ω-Agatoxin IVA, ω-Conotoxin MVIIC	BI-1a BI-1b BI-2a BI-2d	brain,kidney	X57476 (Mori *et al.*,1991) X57688 X57477 X57689
α1B	HVA,N-type	ω-Conotoxin GIVA	α1B-1 α1B-2	brain brain	M94172 M94173
α1C	HVA,L-type	DHP	a,rbC-I b,rbC-II c d	heart,brain heart,brain heart heart	M67516 M67515 J05675 Perez-Reyes *et al.* (1990)
α1D	HVA,L-type	DHP	a b c d	brain, endocrine cells	M76558 Perez-Reyes *et al.* (1990)
α1S	HVA,L-type	DHP	a b	skeletal muscle	M23919 Perez-Reyes *et al.* (1990)
α1E	MVA- P-type	Ni^{2+}	E-1 E-2 E-3 E-4(?)	brain	L29384 L15453 L29385 Williams *et al.* (1994)

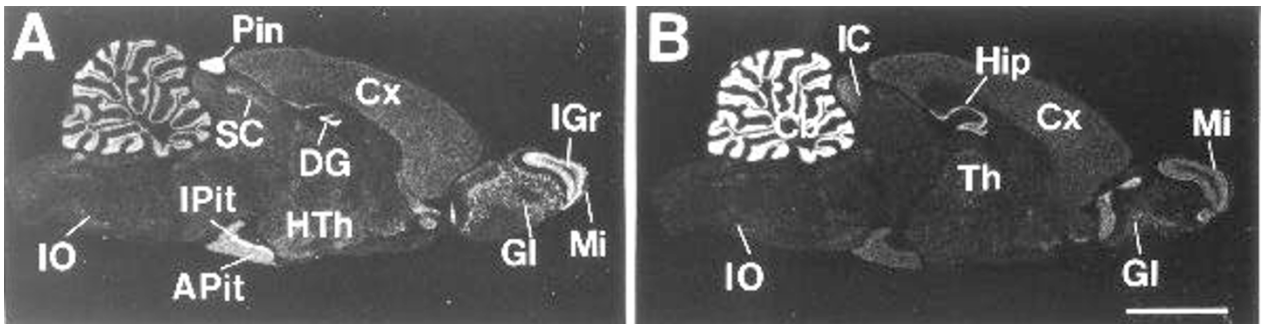

Figure 2. Localization of the L-type α_{1D} (A) and P/Q-type α_{1A} (B) subunits of calcium channels in adult rat brain by *in situ* hybridization. Negative film autoradiography of in situ hybridization of parasagittal sections. Apit, anteriro lobe of pituitary gland; Cb, cerebellum; Cpu, caudate putamen; Cx, cerebral cortex; DG, dentate gyrus; Gl, glomelular layer of olfactory bulb; Hip, hippocampus; HTh, hypothalamus; IC, inferior colliculus; Igr, internal granular layer of olfactory bulb; IO, inferior olive; Ipit, intermediate lobe of pituitary gland; Mi, mitral cell layer; Pin, pineal gland; SC, superior colliculus; Th, thalamus, Bar represents 0.5 cm.

Table 2 Splice variants for the Grenes Encoding α2-δ and β Subunits. Data adapted from Dunlap *et al.*,1995.

Subunits	Gene	Splice Variants	Location(Transcript size)	GenBank Accession No/Reference
α2-δ	α2	α2s	skeletal muscle	M21948
		α2b	brain	M86621
		α2m	Muller glain	Puro *et al* (1996)
β	β1	β1a	skeletal muscle (3.4 kb)	M25817;M92391
		β1b	brain (3,4 kb)	X61394,M92392
		β1c	brain	M92393
	β2	β2a	heart (3.5 kb)	M80545,X64297
		β2b	brain (4 kb)	X64298
		β2c		X64299
		β2d		
	β3	β3a	brain (2.7 kb)	X64300;X76555
		β3b	brain (3 kb)	X76556
		β3c	brain (3.5 kd)	
	β4	β4	cerebellum, kidney (7 kd)	L02315

that multiple VGCC subunit mRNAs are expressed by individual neurons. A recent study of single-cell gene expression in magnocellular neurons in the hypothalamus reveals that nearly all of the known calcium channel subunit transcripts are present, albeit at different levels, in both vasopressin and oxytocin expressing cells (Glasgow *et al*., 1998)

Given the complexity of calcium channel subunits at the molecular level, i.e., the existence of multiple splice variants for the subunits, the combinatorial heterogeneity of the channel subunits expressed in neurons, and the differential cellular and subcellular compartmentalization of theses assemblies (e.g., in dendrites, somata, and axon terminals), it will be essential in the future to correlate the specific α and β subunits forms in a given cell with the specific properties of its calcium currents. Availability of the methods of correlative single-cell gene expression and patch electrode analysis (Eberwine *et al*., 1992; Monyer and Lambolez, 1995) should make such studies feasible.

The regulation of calcium channel gene expression

Molecular insights into how distinct calcium channel subtypes are expressed in a tissue-or cell-type specific manner can be gained by examining mechanisms under-lying regulation of their expression at transcriptional level. In this regard, the restricted expression of the N-type α_{1B} gene in the CNS and wide distribution of the L-type α_{1D} subunit transcript within the CNS and in a variety of other tissues present an

excellent opportunity to examine and compare molecular bases governing calcium channel gene expression. Indeed, the studies on the promoters of the two a_1 subunit gene utilizing a variety of molecular biologic approaches, such as 5'-upstream sequence analysis, deletion analyses using promoter-reporter fusion gene constructs, DNase I foot-printing and electrophoretic mobility assays, have indicated that their expression is differentially regulated at trans-criptional level. The 4.0-kb 5'-flanking sequence of the α_{1B} gene contained a promoter which is capable of directing expression of the α_{1B} transcript in neuronal cells and repressing its expression in non-neuronal cells (Kim *et al*., 1997).Further deletion analysis of α_{1B} subunit-luciferase fusion gene constructs located acis-acting repressor in the distal upstream region. Careful inspection of the sequence in this region did not identify any nucleotide sequence with a significant degree of homology to canonical sequence of the neural-restrictive-silencer-element (NRSE) found in many neuron-specific genes (Schoenherr *et al*., 1996). New negative regulatory elements, in addition to the NRSE, for neural genes have been reported, and future study will reveal identity of a repressor element present on the α_{1B} gene.

The transcription of the L-type α_{1D} gene is regulated by both cis-acting positive and negative elements in the 5' promoter region. Consistent with the broad mRNA expression within and outside of the CNS, a repressor element is not found in the promoter region of the α_{1D} gene. Interestingly, DNase I foot printing, gel mobility shift assay of exon 1 of the α_{1D} gene revealed a DNA binding region that included sequence. This $(ATG)_7$ trinucleotide repeats functions as a novel enhancer *in vitro* when linked to a thymidine kinase promoter and a CAT reporter gene (Kamp *et al*., 1995). Whether or not the $(ATG)_7$ trinucleotide repeats work *in vivo* as an enhancer for the endogenous promoter is an interesting topic for future investigation.

Functions of calcium channels

Influx of calcium through VGCCs localized in different subcellular compartments within the nerve cells mediates a variety of distinct cellular functions in the nervous systems. Calcium ions taken up by the L-type channels on the cell body, through cascades of signaling pathways, are implicated in the regulation of gene expression, whereas increases in localized calcium level in the presynaptic terminals are coupled to excitation-secretion coupling mechanisms. The mechanisms by which calcium influx through VGCCs is linked to neurotransmitter release have been elucidated by identifying proteins that are involved in trafficking, docking and fusion of vesicles, and by characterizing their protein-protein interactions during calcium-evoked exocytosis. These studies have led to a conceptual framework known as the "SNARE" hypothesis (Sollner *et al*., 1993), and extensive homology cloning efforts have yielded very large number of proteins and their respective isoforms with which to construct various models of secretion. Briefly, the term SNARE refers to soluble N-ethylmaleimide-sensitive fusion (NSF) protein receptors which may be present on either vesicles (v-SNARE) or targets (t-SNARE), such as the plasma-memrane. The NSF and SNAPs (soluble NSF attachment proteins, e.g., α, β and γ SNAPs) are believed to be key players in the vesicular docking, priming, and fusion processes (for recent reviews, see Calakos and Scheller 1996; Sudof, 1995; Augustine *et al*., 1996).

ω-conotoxin GVIA-sensitive N-type channels bind to syntaxin, one of the t-SNAREs present on the presynaptic membranes, thereby placing the VGCC in molecular proximity to the proposed SNARE complexes which included SNAP-25 and synaptotagmin (Bennet *et al*., 1992; Leveque, *et al*., 1994). P/Q-type VGCCs have also been shown to co-immunoprecipitate with syntaxin-containing SNARE complexes in rat cerebellar synaptosomes (Martin *et al*., 1996). A peptide motif on N-or P/Q-type VGCC (termed 'synprint' site) that binds to syntaxin in a calcium-dependent manner was found (Sheng *et al*., 1995). A functional consequence of this molecular coupling was subsequently demonstrated in a study where injection of N-type calcium channel synprint peptide into superior cervical ganglion neurons resulted in a dramatic reduction in fast neurotransmitter release (Mochida *et al*., 1996). This interaction appears to have another regulatory function in that co-expression of syntaxin and N- or P/Q-type VGCC in Xenopus oocytes modified gating properties of the expressed channels (Bezprozvanny *et al*., 1995; Weiser *et al*., 1996).

Although much less is known about the molecular mechanisms of peptide and hormone secretion, it is generally believed that the basic mechanisms underlying secretion will be universal and thus the molecular machinery would be conserved between SSV and LDCV secreting systems (Martin, 1994). Diverse isoforms of v-SNAREs and t-SNAREs identified in neurons have shown to be expressed in LDCV containing neuroendocrine and endocrine cells using a combination of RT-PCR, *in situ* hybridization histochemistry, Western Blot, and clostridial toxin analyses. Synaptophysin, rab3b, synaptotagin I/III, munc-18, VAMP-2, CSP, NSF, α-SNAP, SNAP-25, and syntaxin are present in the pituitary gland (Jacobsson and Meister, 1996). VAMP-2, SNAP-25, syntaxin, synaptotagmin III, and munc-18 are also found in pancreatic islets and β cells, and the proteolytic cleavage of VAMP-2 in HIT-T15 cells inhibited calcium-evoked insulin secretion (Wheeler *et al*., 1996).

Diseases linked to calcium channels and mouse mutations with known

defects in calcium channel genes

During the last few years, much progress has been made in understanding the molecular genetic bases for human diseases and mutations in mouse that are linked to defects in calcium channels. They can be divided into three groups; channelopathies that are due to defects in calcium channels in the muscle tissues, those disorders and mutations stemming from abnormality in neuronal calcium channel genes, and autoimmune diseases associated with calcium channels.

Cloning of skeletal muscle a1s subunit gene and subsequent mapping of its location in human and mouse genomes have led to isolation and characterizations of human diseases and mouse mutations caused by skeletal muscle calcium channel defects. Hypokalemic periodic paralysis (HoPP) is associated with three mutations in the a1s subunit gene located on chromosome 1 (1q31 - 32) (Jurkat -Rott *et al*., 1994; Ptacek *et al*., 1994). Two of these affect the outermost argine in the S4 helices of D2 and D4 (R528H and R1239H) accounting for the majority of families with HoPP, while the thrid mutation R1239G is seen rarely. The lethal autosomal recessive mutation muscular dysgenesis (mdg) phenotype, which results in total lack of excitation-contraction coupling in all homozygous dysgenic offspring (Pai, 1965), can be rescued by microinjection of a cloned α_{1s} subunit cDNA in noncontracting mdg/mdg primary muscle cultures (Tanabe *et al*., 1988). *Mdg* mutation is caused by mutations in the α_{1s} subunit gene located on mouse chromosome 1 (Chin *et al*., 1992b).

Molecular genetic studies of neuronal VGCCs have yielded evidence for a number of interesting mechanisms that link channel gene defects to diseases of mice and men. The best-characterized cases, thus far, is those channelopathies associated with the P/Q-type α_{1A} subunit gene. Ophoff and his colleagues (1996) cloned a gene on chromosome 19, which are linked to the two rare forms of human neurological disorders: familial hemiplegic migraine (FHM) and episodic ataxia type-2 (EA-2). FHM is a rare autosomal dominant subtype of migraine, which is associated with, in some families, progressive cerebellar atrophy. EA-2 is another autosomal dominant paraxysmal cerebral disorder, which is characterized by cerebellar ataxia migraine-like symptoms, and cerebellar atrophy. Mis-sense mutations scattered along the entire coding region are found in individuals with FHM and splice site mutation in EA-2 patients, which leads to a truncated α_{1A} subunit protein. More recently, a slowly progressing autosomal dominant form of cerebellar ataxia, termed spinocerebellar ataxia type 6 (SCA6), is found to be associated with CAG trinucleotide expansion in the a1A subunit gene (Zhuchenko *et al*., 1997). A five-base pair (GGCAG) insertion prior to the previously detected stop codon in combination with a 36-base pair deletion in the a1A subunit gene would produce a diverse molecular forms of α_{1A} protein whose 3' carboxy-termini extended beyond the normal α_{1A} subunit protein. It would be of great interest to examine biophysical properties of calcium channels expressed by these extended versions of the α_{1A} subunit gene and their possible roles in normal and pathophysiological neuronal functions.

The mouse mutant phenotype of tottering (*tg*) and leaner are associated with defects in the α_{1A} subunit gene. The *tg* mice exhibit severe ataxia, Petit-mal-like epilepsy and a myoclonus-like movement disorder, and the leaner mutation in an allele of *tg* (tg^{la}/tg^{la}) causes profound chronic ataxia associated with cerebellar atrophy resulting from pervasive loss of Purkinje and granule cells. These mutants have been found to carry an autosomal recessive mutation in a splice donor consensus sequence in the α_{1A} subunit gene, which is not surprising in that the P/Q-type channels are the most abundant VGCC subtype expressed in cerebellar Purkinje and granule cells. The aberrant splicing of intron/exon junctions results in truncation of the normal open reading frame and is thought to give rise to two α_{1A} splice variants with novel carboxy-terminal sequence (Fletcher *et al*., 1996). Examination of α_{1A} transcript and protein expression, by *in situ* hybridization and immunocytochermistry, in the leaner and age-matched control mice show no quantitative differences in their levels in cerebellar Purkinje and granule cells (Lau *et al*., 1998). HVA calcium currents carried by P-type channels, however, are reduced in acutely isolated leaner (tg^{la}/tg^{la}) Purkinje cells exhibit as compared to Purkinje cells from age-matched control mice (Dove *et al*., 1997; Lorenzon, *et al*., 1998). The specific cause for the reduction in tg^{la}/tg^{la} Purkinje cells calcium current remains to be determined.

In addition to the α_{1A} and α_{1s} genes, there are indications that other pore-forming a1 subunits (i.e., α_{1B} or α_{1E}) and ancillary β subunit might all play a role in other disease states in human and mouse mutations. For example, the Lambert-Eaton myasthenic Syndrome (LES) is an autoimmune disorder of neuromuscular transmission, of which clinical phenotypes include muscle weakness caused by reduction in acetylcholine release from presynaptic terminals innervating muscle tissues. As shown in other congenital myasthenic syndromes such as Myasthenia gravis and acquired neuromyotonia which are also often associated with Thyoma, LES is often associated with small-cell lung carcinoma (SCLC). This observation probably reflects the host immune response against components on tumor cells. The circulating antibodies against α_{1A} subunit protein, and to a lesser degree, β_{1B} subunit protein of the N-type calcium channels, are found in LES patients (Lennon *et al*., 1995). Moreover, antibodies directed against the peptide corresponding to the unique acidic stretch in the IV S5-S6 linker region of α_{1A} subunit, inhibited calcium currents in SCLC, suggesting that a

possible important role for α_{1A} calcium channels in the pathogenesis of LES.

The first clue that β_4 subunit gene is linked to lethargic mutation in mouse has come from a genetic mapping study showing that mouse β_4 gene maps closely to lethargic locus on chromosome 2 (Chin *et al*., 1995). Subsequently, it has been shown that there is a four-nucleotide insertion in β_4 subunit gene in the lethargic mouse resulting in a splicing error that give rise to a severely truncated β_4 protein (Burgess *et al*., 1997). The β_4 subunit is present abundantly in cerebellum, and temporal pattern of its mRNA expression closely resemble that of α_{1A} transcripts (Kim and Chin, 1997). This truncated β_4 subunit protein, which contains ~40% of the amino-terminal portion, is missing the site that interacts with the a1A subunit proteins, thus is unable to form fully functional channels. Lack of mature β_4 protein in lethargic mouse might result in dysfunctional calcium channels in cerebellar Purkinje and granule cells, and thus underlie observed phenotypes of ataxia and bsence-like seizures, similar to the tottering mouse.

Future Direction

While a diversity of neuronal calcium channel had been appreciated from electrophysiological analyses of VGCCs found in excitable cells, the genetic and biochemical diversity of these molecules has proven greater than anticipated. With this expanded knowledge of the structure and function of neuronal VGCCs, we are well poised to devise innovative strategies not only to identify novel calcium channel subtypes and their specific inhibitors, but also to define the molecular determinants of functional modulation of the channels.

With regard to the first point, there is no other instance that the power and utility of genome sequencing initiative is better exemplified than the recent cloning of a T-type calcium channels (Perez-Reyes *et al*., 1998). In contrast to HLA calcium channels, the molecular entity of LVA T-type calcium channels, despite of their well-characterized biophysical properties, has been a subject of intense discussion. While some suggested that α_{1E} gene constitutes a pore-forming subunit of a LVA channel based on their expression studies in Xenopus oocytes (Soong *et al*., 1993), others reported that LVA currents are expressed in mouse neuroblastoma cell lines in the absence of α_{1E} mRNA, suggesting that these channels comprise a distinct molecular class (Gottschalk *et al*., 1996). Perez-Reyes and his colleagues have taken a different cloning approach that utilized the wealth of information available in the Caenorhabditis elegans genome database. Search of C. elegans expressed sequence tag (EST) database with a homology probe derived from a highly conserved region of calcium channel a1 subunits has led them to identification and cloning of a novel family of cDNAs encoding LVA T-type calcium channels. Isolation of this T-type channel cDNA undoubtedly will lead to isolation of a new and important family of LVA VGCCs and identifi-cation of a new class of pharmacological inhibitors and therapeutical reagents specific for the T-type channels. Availability of genomic databases and genetic mapping databases will provide new research tools for channel discovery and analysis of channel functions that are complementary to standard"wet bench approach".

A second area of research in calcium channel field will continue to be elucidation of molecular and cellular bases of calcium channel functions. We still do not fully understand how distinct calcium channel subtypes are assembled and targeted to different cellular compartments (soma, dendrites, and axon terminals), Nor do we know how calcium channel subtypes play key roles in signal transduction and plasticity in developing, mature, and aging nervous systems through interactions with other cellular proteins.

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