Structure-Functional Diversity of Human L-Type Ca\textsuperscript{2+} Channel: Perspectives for New Pharmacological Targets

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ABSTRACT

The L-type Ca\textsuperscript{2+} channels mediate depolarization-induced influx of Ca\textsuperscript{2+} into a wide variety of cells and thus play a central role in triggering cardiac and smooth muscle contraction. Because of this role, clinically important classes of 1,4-dihydropyridine, phenylalkylamine, and benzothiazepine Ca\textsuperscript{2+} channel blockers were developed as powerful medicines to treat hypertension and angina pectoris. Molecular cloning studies revealed that the channel is subject to extensive structure-functional variability due to alternative splicing. In this review, we will focus on a potentially important role of genetically driven variability of Ca\textsuperscript{2+} channels in expression regulation and mutations, Ca\textsuperscript{2+}-induced inactivation, and modulation of sensitivity to Ca\textsuperscript{2+} channel blockers with the perspective for new pharmacological targets.

The L-type Ca\textsuperscript{2+} channel is a ubiquitously expressed voltage-gated ion channel supporting inward current of Ca\textsuperscript{2+} ions that play a central role as an intracellular second messenger in many processes ranging from gene expression to cardiac and smooth muscle contraction. A number of L-type Ca\textsuperscript{2+} channel blockers have been developed for the treatment of cardiovascular disorders. 1,4-Dihydropyridines, which have proven to be the most potent inhibitors of the channel, were used for its biochemical identification in rabbit skeletal muscle T-tubules as a protein complex composed of the pore-forming \(\alpha\text{1S}\) subunit and auxiliary \(\alpha\text{2}\delta\), \(\beta\), and \(\gamma\) subunits. Two dihydropyridine-sensitive genetic variants of \(\alpha\text{1S}\) have been identified and cloned from heart (\(\alpha\text{1C}\)) and pancreatic beta cells (\(\alpha\text{1D}\)), with the cardiac \(\alpha\text{1C}\) isofrom being expressed in the vast majority of eukaryotic cells. Activity of the \(\alpha\text{1C}\) channel is highly regulated by membrane potential, other calcium channel auxiliary subunits (\(\alpha\text{2}\delta\)), and by feedback dependence on permeating Ca\textsuperscript{2+}, which is mediated by calmodulin. The genetic regulation of the \(\alpha\text{1C}\) Ca\textsuperscript{2+} channel subunit occurs through species-specific variability and largely tissue-specific alternative splicing. In this review, we briefly discuss genomic organization of the \(\alpha\text{1C}\) subunit gene and then focus attention on alternative splicing that generates functionally distinct Ca\textsuperscript{2+} channel isoforms as potentially new pharmacological targets.

Genomic Organization

The \(\alpha\text{1C}\) L-type Ca\textsuperscript{2+} channel is subject to complex genetic regulation that gives rise to a variety of channel subtypes. Both genomic variability and alternative splicing of the primary transcript appear to contribute to this complexity. The human \(\alpha\text{1C}\) subunit gene (CACNL1A1) is composed of 53 identified exons (Fig. 1) (Soldatov, 1994) and is located in the distal region of chromosome 12p13 (Sun et al., 1992; Powers et al., 1992). There are two other \(\alpha\) subunits (\(\alpha\text{1D}\) and \(\alpha\text{1S}\)) of the L-type Ca\textsuperscript{2+} channel subfamily that are encoded by different genes located in different chromosomes.

The exact size of the \(\alpha\text{1C}\) subunit gene remains to be determined. Based on the assumption that 10 kb is a rough size approximation of unknown introns, the gene was estimated to span more than 150 kb of the human genome (Soldatov, 1994). However, the high-resolution visual mapping of stretched DNA by fluorescent in situ hybridization (termed fiber-FISH) has shown that the distance from exon 10 to the 3’-end is in fact 170.4 kb (Liu et al., 1998). Thus the total length of the human CACNL1A1 gene is at least 70 kb greater than earlier estimates. In addition, we have found a repetitive element of three paired exon 45/46-related sequences in the \(\alpha\text{1C}\) subunit gene (Soldatov et al., 1998). Two of them, clones g6-20 and g12-5, were found by the fiber-
Fig. 1. Hypothetical transmembrane topology of the polypeptide chain of the channel-forming α_{1C} subunit of the human L-type Ca^{2+} channel. Shown are four repeats (I, II, III, IV) each consisting of six putative transmembrane segments. Both NH₂ and COOH termini are in the cytoplasm. Transmembrane segments S4 contain from 3 to 5 positively charged amino acid residues and presumably form the voltage sensor of the channel. Bold bars mark segments encoded by numbered exons. Arrows point to transmembrane segments encoded by alternative exons 7, 33, and 45, which are subject to constitutive splicing (Soldatov, 1992, 1994). Boxed are the Ca^{2+}-sensing domains L (1572–1598) and K (1599–1615) coded by alternative exons 40 to 42 that are subject to alternative splicing leading to α_{1C,66} (Soldatov et al., 1997). Motifs involved in Ca^{2+}-induced inactivation are shown in bold letters: M1 (1572–1576), M8 (1580–1587), M3 (1600–1604), and M4 (1630–1634). The combined M1 + M3 mutant was found to be deprived of Ca^{2+}-induced inactivation similar to α_{1C,77l}, α_{1C,77k} (Soldatov et al., 1998), and α_{1C,86} (Soldatov et al., 1997). Amino acids that did not contribute to Ca^{2+}-induced inactivation are shown in italics. The Ca-binding IQ motif (1624–1635) (Zühlke and Reuter, 1998) is underlined. The full-length domain L (1572–1599) and the sequence encompassing M3 and M8 (1582–1604) both show high-affinity CaM-binding in low (50 nM) and high (2 μM) [Ca^{2+}]_{free}. The high-affinity Ca^{2+} sensor has been identified as sequence 1572–1587 (Romain et al., 2000). A larger panel of sequences from similar regions of other channel types has been investigated by Pate et al. (2000).

FISH technique to be located within 59.1 kb downstream of the polyadenylation site, i.e., 230 kb away from exon 10. Thus, the total size of the human CACNL1A1 gene may be 300 kb.

The idea that the α_{1C} gene contains several repetitive 3′-terminal sequences is indirectly supported by the fact that cDNA coding for human and nonhuman Ca^{2+} channel α_{1C} subunits significantly diverge downstream from exon 44. This divergence may originate from variability in the 3′-terminal part of the gene. In the absence of data indicating that the 3′-end of CACNL1A1 may be subject to alternative splicing, we hypothesize that sequences representing g12-5 and g6-20 could become silent during evolution of this very complex gene.

A new exon 45/46-related sequence g8-19 has been identified in the human genome and mapped by FISH to the 12p11.2 and 12p13.2-p13.1 bands (Soldatov et al., 1998b). These positions were not recognized by DNA probes generated from the 5′- and 3′-terminal regions of the α_{1C} gene. It is possible that hybridization of g8-19 to two loci occurs because 1) this DNA may in fact be a chimeric clone that contains sequences from 12p11.2 and 12p13.2-p13.1 that are not normally contiguous or 2) g8-19 may contain repetitive elements other than the exon 45/46-related sequence, which are recognized by both loci. However, given the unique similarity of its exon 45/46-related sequences to the α_{1C} gene, an alternative hypothesis is that g8-19 belongs to a new gene or pseudogene of the same α_{1} family.

Promoters and Expression Regulation

Human α_{1C} transcripts are highly homologous to those from mice, rat, and rabbit except exons 17 and 44–50. Exon 1 comprising an initiation codon and the 5′-untranslated region also appears to be subject to alternative splicing. Indeed, the α_{1C} transcripts identified in rabbit heart (Mikami et al., 1989) and rat aorta (Koch et al., 1990) have exon 1 (subsequently referred to as exon 1A) different from α_{1C} transcripts in rabbit lung (Biel et al., 1990), rat heart (Schunk et al., 1991), human fibroblasts (Soldatov, 1992), and hippocampus (N. M. Soldatov, unpublished observation) thus suggesting that both exon 1 isoforms of α_{1C} are not species-but rather tissue-specific. Unlike these two isoforms, the human heart α_{1C} cloned by Schultz et al. (1993) has exon 1 and an upstream part of exon 2 deleted. Since the putative splice acceptor site that would be employed in this case does not conform to the consensus sequence, the proposed shortned N terminus of this α_{1C} isoform must be validated by genomic DNA sequences. In fact, exon 1A with the respective 5′-untranslated region appears to be present in human chromosome 12 at the genomic DNA region upstream of the identified exon 1 (N. Dascal, personal information). This exon contributes to the α_{1C} transcripts in human cardiac tissue.

The promoter region for the exon 1A isoform of rat α_{1C} (Mikami et al., 1989; Koch et al., 1990) lacks a canonical TATA sequence but has a consensus Inr element corresponding to the major transcription start site (Liu et al., 2000). Seven possible additional transcription initiation sites were identified within a 100-nt distance around Inr, as well as a number of potential regulatory elements in a 2-kb sequence upstream of the major 5′-cap site. Those elements included five consensus sequences for transcription factor Nkx2.5, which is specific for developing heart and exhibits modest transcriptional activation. Other elements included the potentially important hormone-responsive elements associated with response to steroids, the CAM-responsive element, and AP-1 that is regulated by mitogen-activated protein kinase signal transduction pathways. Other elements identified are two muscle determination factors MyoD and MEF2 and several other putative elements including C/EBPh, Oct-1, GRE, NF-E3/C-Ets, and STATX.
The effect of some of these factors on α1C transcripts and protein levels has been experimentally documented. As determined by dihydropyridine (DHP) binding assay, serum deprivation increased the amount of α1C channels in human fibroblasts that returned to baseline when serum was reintroduced (Dudkin et al., 1988). The individual mitogens including epidermal growth factor, basic fibroblast growth factor, and insulin reduced the amount of DHP receptors in cells (Soldatov et al., 1988). In cardiac myocytes, incubation in high Ca2+ increased both transcription of α1C channels and DHP binding (Davidoff et al., 1997). In contrast, exposure to phenylephrine, an α1 adrenergic agonist with signal transduced as cytosolic Ca2+ elevation via receptor-operated calcium channels, decreased α1C transcripts and L-type calcium currents (Maki et al., 1996). Isoproterenol and β-bromocAMP increased Ca2+ channel mRNA and peak calcium current (Maki et al., 1996). These findings all support a role for the α1C Ca2+ channel in mitogenic responses and cellular proliferation. Whether the opposite effects of norepinephrine-mediated increase of [Ca2+]i, via receptor-operated Ca2+ channels compared with direct increase of [Ca2+]i in the medium are due to specific involvement of a different set of regulatory elements remains to be studied.

Fifteen exons of the human α1C gene have been established to be subject to alternative splicing. The regulation of Ca2+ channel expression through alternative splicing (Perez-Reyes et al., 1990) is particularly interesting in terms of regional structural diversity. Alternative splicing affects regions encoding transmembrane segments IIIS2, IVS3, as well as the intracellular C-terminal tail and linkers between repeats I, II, and III. Systematic study of the expression of α1C splice variants has not yet been undertaken but may soon become possible with the development of new proteomic methods such as matrix-assisted laser desorption/ionization mass spectrometry. Limited data available at this time indicate that alternative splicing of α1C may occur in a species- and tissue-specific fashion and can generate channel isoforms with altered functional properties. One of these splicing events at transmembrane IVS3 is related to development of Ca2+ channel isoform expression (Diebold et al., 1992). Our preliminary data (Soldatov et al., 2001) show that switch of the exon 22 to 21 isoform of α1C occurs in response to suppression of proliferative stimuli in human aortic smooth muscle in an age-specific manner with cells from donors older than about 50 years expressing exon 21 and cells from younger donors not expressing exon 21. However, only few alternative isoforms of α1C show properties of functionally distinct Ca2+ channel subtypes.

Ca2+ Sensors of the Ca2+ Channel

Useful information to help understand the molecular bases of Ca2+-dependent regulation of the L-type Ca2+ channel activity has been obtained from studies of alternative exons 40–43 that were identified in a partial α1C transcript of human hippocampus (Soldatov, 1994). Reconstruction of the alternative exons into the α1C-coding sequence of the conventional (α1C,77) channel has resulted in a Ca2+-insensitive isoform (α1C,86) of the channel (Soldatov et al., 1997) with 80 amino acids in the second quarter of the cytoplasmic tail of the channel replaced by 81 nonidentical amino acids (Fig. 1). α1C,86 conducted Ba2+ and Ca2+ currents with almost identical fast kinetics of inactivation and did not show a U-shape dependence of the time course of inactivation on membrane voltage, which is the characteristic feature of Ca2+-induced inactivation. By replacement of large segments of this 81-amino acid domain of α1C,86 into the α1C,77 channel, it was found (Soldatov et al., 1998a) that Ca2+-induced inactivation is independently determined by two sequences that we have called domains L and K (Fig. 1). Ca2+-induced inactivation was found to partially depend on several shorter sequences identified in these domains (shown in Fig. 1 in bold letters). Only their combined mutation within or between domains L and K removed the Ca2+ sensitivity of the channel inactivation. Some of the identified important partial motifs have been later confirmed by deletion analysis (Zühlke and Reuter, 1998).

Both domains L (Pate et al., 2000; Romanin et al., 2000) and K (Peterson et al., 1999; Qin et al., 1999; Zühlke et al., 1999) were found to bind calmodulin (CaM) involved in Ca2+-induced inactivation. In addition, domain L contains a highly specific Ca2+ sensor ($K_a \approx 100 nM$) composed of motifs M1 and M8, both of which are important for Ca2+-dependent inactivation of the channel. Ca2+ loading of this Ca2+ sensor was shown to modulate the CaM affinity of CaM-binding site in domain L. The IQ motif in domain K (Fig. 1) appears to have a role in the binding of Ca2+–loaded CaM. Translocation or sliding of CaM with possible involvement of motif M3 or independent binding of CaM to the two sites appears to regulate different stages of the channel activity.

It is known that Ca2+-saturated CaM can adopt different conformations upon interaction with CaM-binding domains, from an extended dumbbell shape to a globular shape wrapped around the target peptide (Elshorst et al., 1999). There are examples when either one or both Ca2+-binding halves of the CaM molecule are needed for activation, or their binding occurs with no effect. Whether CaM is able to form a triple complex with two discrete determinants in L and K (Mouton et al., 2001), or they represent distinct or alternative binding sites (Pate et al., 2000) remains to be verified by direct structural studies. The constitutive nature of CaM binding, however, explains why CaM inhibitors did not affect Ca2+-induced inactivation in earlier studies (Zühlke and Reuter, 1998).

The human α1C channel did not show current facilitation by strong positive voltage prepulses (N. M. Soldatov and H. Reuter, unpublished observation). Interestingly, the I1604A mutation in the IQ motif of α1C,77 revealed CaM-dependent facilitation of Ca2+ but not Ba2+ current in response to an applied train of high-frequency depolarizations (Zühlke et al., 1999). This property was unmasked in cardiac myocytes by intracellular application of a domain L-derived peptide (1579–1604) (Pate et al., 2000). Collectively, these data suggest a new principle of modulatory control over Ca2+ signaling mediated by the α1C channel particularly in cardiac and vascular cells.

The cross-talk between two CaM-binding sites is transduced into Ca2+-driven inactivation by a yet unknown mechanism. Replacements in the cytoplasmic 80-amino acid segment leading to α1C,76, α1C,77L, and α1C,77K variants affected voltage sensors for activation and inactivation (Soldatov et al., 1997, 1998a), and reduced open probability and single channel conductance (Kepler et al., 2000b) thus suggesting that both Ca2+-sensing domains interact with a pore
region and affect the voltage-gating mechanism of the channel. Combined disruption of motifs L and K in \( \alpha_{1C,86} \) completely eliminated the characteristic run-down property of the channel (Keppler et al., 2001). This region may involve determinants for the rescue effect of calpastatin (Romanin et al., 1991).

Increasing evidence demonstrates clustering of recombinant channels in the plasma membrane of expressing cells. By the photobleaching of \( \alpha_{1C,77} \) N-terminally labeled by enhanced yellow fluorescent protein, it was found that a mean cluster contains approximately 40 channels (Harms et al., 2001). Application of label-fracture and cryothin-sectioning techniques with high-resolution immunogold-labeling to guinea pig ventricular myocytes allowed direct demonstration of \( \alpha_{1C} \) channels clustering, which occurred predominantly in plasma membrane domains overlying junctional sarcoplasmic reticulum (Gathercole et al., 2000). Therefore, membrane targeting and subsequent clustering appear to be important features of \( \alpha_{1C} \) for its function as a trigger of intracellular \( \text{Ca}^{2+} \) release involved in excitation-contraction coupling in cardiac muscle. We found that determinants for both features are located in the same 80-amino acid segment of the tail (1572–1651). Indeed the \( \alpha_{1C,86} \) channel labeled N-terminally by green fluorescent protein was predominantly distributed within the cytoplasm. Only a minor fraction of \( \alpha_{1C,86} \) was able to incorporate into the plasma membrane, and this may correspond to low current density that is generally observed upon \( \alpha_{1C,86} \) expression (Keppler et al., 2000b). The membrane-bound fraction of the labeled \( \alpha_{1C,86} \) did not form characteristic clusters.

**Modulation of Sensitivity to DHP**

Free \( \text{Ca}^{2+} \) is known to be important for the high-affinity interaction of \( \text{Ca}^{2+} \) channels with DHP calcium channel blockers. However, disruption of \( \text{Ca}^{2+} \) sensors increased DHP sensitivity of \( \alpha_{1C,86} \) 3.5-fold compared with \( \alpha_{1C,77} \) over a wide range of potentials (Soldatov et al., 1997; Zühlke et al., 1998). Thus, the modulation of DHP sensitivity observed in \( \alpha_{1C,86} \) occurs in a \( \text{Ca}^{2+} \)-independent manner.

An opposite but also voltage-independent modulation of the sensitivity to isradipine was found to be caused by a replacement of exon 8 for 8A (Fig. 1) leading to the \( \alpha_{1C,105} \) isoform with a modified transmembrane segment IS6 (Zühlke et al., 1998). Unlike the \( \alpha_{1C,86} \), \( \alpha_{1C,72} \), and \( \alpha_{1C,105} \) channels, \( \alpha_{1C,70} \) produced by substitution of exon 22 for 21, showed an altered voltage-dependent inhibition of \( \text{Ba}^{2+} \) current by isradipine at very negative potentials (Soldatov et al., 1995). The slope of the IC\text{so}_{50} \) curve at \( \approx 90 \) mV was significantly less steep in \( \alpha_{1C,70} \) than in \( \alpha_{1C,77} \), causing a 2.5-fold difference in the inhibitory potency of the drug between the two channels. These results suggest that the external portion of the putative transmembrane segment III2, encoded by exons 21 and 22, experiences voltage-dependent conformational changes that alter DHP binding. The voltage dependence of isradipine action is more pronounced in the exon 21 than in the exon 22 isoform of the channel.

The identified sites of modulation for isradipine inhibition are located in different regions of \( \alpha_{1C} \) but outside of the high-affinity binding site for DHPs. These altered pharmacological properties of the \( \alpha_{1C} \) channel isoforms imply that alternative splicing may contribute to the tissue specificity and to age-related changes in the clinical effects of DHP calcium antagonists, at least in vascular smooth muscle (Ab ernethy and Schwartz, 1999).

**Truncated Forms and Mutants of \( \alpha_{1C} \)**

Screening of brain and fibroblast transcripts has revealed a dominant truncated form of the human \( \alpha_{1C} \) calcium channel subunit, which originates from the utilization of an alternative splice acceptor site at the 5'-end of exon 15, conforming better for splice acceptor requirements than the functional one (Soldatov, 1994). This leads to a 73-base pair deletion and interruption of the reading frame in the region of transmembrane segment IIIS6 in as many as 75% of \( \alpha_{1C} \) transcripts. The role of this and the other much less abundant truncated form produced by a 12-nt insertion at the 3'-end of exon 16 remains to be clarified. Recently, two proteins produced by truncations of the \( \alpha_{1C} \) gene in the region of exons 17–19 have been identified in rabbit sarcolemma and sarcoplasmic reticulum (Wielowieyski et al., 2001). These forms may have a role in excitation-contraction coupling or in sequestering auxiliary \( \beta \) subunits by the \( \alpha_{1C,\beta} \) interaction site that is retained in the repeat I–II linker.

Structure-functional studies of naturally occurring mutations that affect \( \text{Ca}^{2+} \) channel properties are of particular interest as they may allow identification of new therapeutic targets. One such mutation was originally identified as a single nucleotide conversion \( g^{2254} \rightarrow a \) in two independent human fibroblast \( \alpha_{1C} \) subunit transcripts (Soldatov, 1992). This produced substitution by Thr of the invariant Ala752 residue located at the cytoplasmic end of the highly conserved transmembrane segment IIIS6, which significantly impaired voltage-gated inactivation (Soldatov et al., 2000). Such a “leaky” mutant may cause \( \text{Ca}^{2+} \) overload of the cell and cytotoxicity, however, this remains to be proven.

The remarkable structure-functional diversity of the \( \alpha_{1C} \) calcium channel requires further systematic investigation. An interesting approach for exploration of functional links in calcium channel regulation was gained with coexpression of \( \text{Ca}^{2+} \) channel isoforms with receptors that have in vivo functional interaction with the \( \text{Ca}^{2+} \) channel. For example, coexpression of the \( \alpha_{1C,77} \) channel with the angiotensin type IA receptor in \( \text{Xenopus} \) oocytes allowed study of regulation of the L-type \( \text{Ca}^{2+} \) channel by angiotensin. This regulation was mediated via IP3-induced intracellular \( \text{Ca}^{2+} \) release and occurred at the molecular motif responsible for the \( \text{Ca}^{2+} \)-induced inactivation of the channel (Oz et al., 1998). Use of diverse functional isoforms of \( \text{Ca}^{2+} \) channel as biosensors and the measurement of voltage-gated \( \text{Ca}^{2+} \) and \( \text{Ba}^{2+} \) currents in such systems offers new opportunities to investigate pharmacological properties of coexpressed receptors and to study the mechanism of in vivo drug effects. In this particular example, these findings were helpful in understanding a vascular interaction between angiotensin II and calcium antagonist drugs seen in clinical study (Andrawis et al., 1992).

Recent discoveries of the molecular bases of \( \text{Ca}^{2+} \) channel inactivation mechanisms, particularly of its \( \text{Ca}^{2+} \) dependen
dence, and their evolving role in excitation-contraction coupling in cardiac and vascular cells point to a necessity of detailed structural investigation of the involved regions, particularly bearing \( \text{Ca}^{2+} \) sensors, using diffraction and NMR methods. Careful investigation of intramolecular protein-
protein interactions critical for activation and termination of Ca\(^{2+}\) current will obviously help develop new therapeutic targets based on new principles.

References


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