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

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ABSTRACT

The L-type Ca\(^{2+}\) channels mediate depolarization-induced influx of Ca\(^{2+}\) 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-dihydropyridines, phenylalkylamine, and benzothiazepine Ca\(^{2+}\) 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\(^{2+}\) channels in expression regulation and mutations, Ca\(^{2+}\)-induced inactivation, and modulation of sensitivity to Ca\(^{2+}\) channel blockers with the perspective for new pharmacological targets.

Genomic Organization

The \(\alpha_{1c}\) L-type Ca\(^{2+}\) 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 \(\alpha_{1c}\) 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 \(\alpha_{1}\) subunits (\(\alpha_{1D}\) and \(\alpha_{1S}\)) of the L-type Ca\(^{2+}\) channel subfamily that are encoded by different genes located in different chromosomes.

The exact size of the \(\alpha_{1c}\) 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 \(\alpha_{1c}\) subunit gene (Soldatov et al., 1998b). Two of them, clones g6-20 and g12-5, were found by the fiber-

ABBREVIATIONS: kb, kilobase(s); FISH, fluorescent in situ hybridization; DHP, dihydropyridine; CaM, calmodulin; nt, nucleotide(s).
Both show high-affinity CaM-binding in low (50 nM) and high (2 μM) Ca2+-induced inactivation. The CaM-binding IQ motifs involved in Ca2+-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 Ca2+-induced inactivation similar to α1C<sub>T1L8</sub> α1C<sub>T1T8</sub> (Soldatov et al., 1998b). Amino acids that did not contribute to Ca2+-induced inactivation are shown in italics. The CaM-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) [Ca2+]<sub>i</sub>. The high-affinity Ca2+ sensor has been identified as sequence 1572–1587 (Romanin 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 α1<sub>C</sub> gene contains several repetitive 3’-terminal sequences is indirectly supported by the fact that cDNA coding for human and nonhuman Ca2+-channel α1<sub>C</sub> 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 α1<sub>C</sub> 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 α1<sub>C</sub> gene, an alternative hypothesis is that g8-19 belongs to a new gene or pseudogene of the same α1 family.

**Promoters and Expression Regulation**

Human α<sub>1C</sub> 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<sub>1C</sub> 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<sub>1C</sub> transcripts in rabbit lung (Biel et al., 1990), rat heart (Schnuch et al., 1991), human fibroblasts (Soldatov, 1992), and hippocampus (N. M. Soldatov, unpublished observation) thus suggesting that both exon 1 isoforms of α<sub>1C</sub> are not species- but rather tissue-specific. Unlike these two isoforms, the human heart α<sub>1C</sub> 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 shortened N terminus of this α<sub>1C</sub> 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<sub>1C</sub> transcripts in human cardiac tissue.

The promoter region for the exon 1A isoform of rat α<sub>1C</sub> (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 steroid hormones, the cAMP-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 α_{1C} transcripts and protein levels has been experimentally documented. As determined by dihydropyridine (DHP) binding assay, serum deprivation increased the amount of α_{1C} 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 Ca^{2+} increased both transcription of α_{1C} channels and DHP binding (Davidoff et al., 1997). In contrast, exposure to phenylephrine, an α\_1 adrenergic agonist with signal transduced as cytosolic Ca^{2+} elevation via receptor-operated calcium channels, decreased α_{1C} transcripts and L-type calcium currents (Maki et al., 1996). Isoproterenol and 8-bromo-cAMP increased Ca^{2+} channel mRNA and peak calcium current (Maki et al., 1996). These findings all support a role for the α_{1C} Ca^{2+} channel in mitogenic responses and cellular proliferation. Whether the opposite effects of norepinephrine-mediated increase of [Ca^{2+}], via receptor-operated Ca^{2+} channels compared with direct increase of [Ca^{2+}], in the medium are due to specific involvement of a different set of regulatory elements remains to be studied.

Fifteen exons of the human α_{1C} gene have been established to be subject to alternative splicing. The regulation of Ca^{2+} 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 IIIIS2, IVS3, as well as the intracellular C-terminal tail and linkers between repeats I, II, and III. Systematic study of the expression of α_{1C} 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 α_{1C} 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 developmental regulation of Ca^{2+} 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 α_{1C} 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 α_{1C} show properties of functionally distinct Ca^{2+} channel subtypes.

**Ca^{2+} Sensors of the Ca^{2+} Channel**

Useful information to help understand the molecular bases of Ca^{2+}-dependent regulation of the L-type Ca^{2+} channel activity has been obtained from studies of alternative exons 40–43 that were identified in a partial α_{1C} transcript of human hippocampus (Soldatov, 1994). Reconstruction of the alternative exons into the α_{1C}-coding sequence of the conventional (α_{1C,77}) channel has resulted in a Ca^{2+}-insensitive isoform (α_{1C,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). α_{1C,86} conducted Ba^{2+} and Ca^{2+} 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 Ca^{2+}-induced inactivation. By replacement of large segments of this 81-amino acid domain of α_{1C,86} into the α_{1C,77} channel, it was found (Soldatov et al., 1998a) that Ca^{2+}-induced inactivation is independently determined by two sequences that we have called domains L and K (Fig. 1). Ca^{2+}-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 Ca^{2+} 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; Romain 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 Ca^{2+}-induced inactivation. In addition, domain L contains a highly specific Ca^{2+} sensor (K_{0.5} ∼ 100 nM) composed of motifs M1 and M8, both of which are important for Ca^{2+}-dependent inactivation of the channel. Ca^{2+} loading of this Ca^{2+} 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 Ca^{2+}-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 Ca^{2+}-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 Ca^{2+}-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 Ca^{2+}-induced inactivation in earlier studies (Zühlke and Reuter, 1998).

The human α_{1C} channel did not show current facilitation by strong positive voltage prepulses (N. M. Soldatov and H. Reuter, unpublished observation). Interestingly, the I1624A mutation in the IQ motif of α_{1C,77} revealed CaM-dependent facilitation of Ca^{2+} but not Ba^{2+} 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 Ca^{2+} signaling mediated by the α_{1C} channel particularly in cardiac and vascular cells.

The cross-talk between two CaM-binding sites is transduced into Ca^{2+}-driven inactivation by a yet unknown mechanism. Replacements in the cytoplasmic 80-amino acid segment leading to α_{1C,86}, α_{1C,77L}, and α_{1C,77K} variants affected voltage sensors for activation and inactivation (Soldatov et al., 1997, 1998a), and reduced open probability and single channel conductance (Keplinger et al., 2000b) thus suggesting that both Ca^{2+}-sensing domains interact with a pore
region and affect the voltage-gating mechanism of the channel. Combined disruption of motifs L and K in α1C,86 completely eliminated the characteristic run-down property of the channel (Keppeler et al., 2000a). This region may involve determinants for the rescue effect of calpastatin (Roman et al., 1991).

Increasing evidence demonstrates clustering of recombinant channels in the plasma membrane of expressing cells. By the photobleaching of α1C,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 cryo-thin-sectioning techniques with high-resolution immunogold-labeling to guinea pig ventricular myocytes allowed direct demonstration of α1C 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 α1C for its function as a trigger of intracellular 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 α1C,86 channel labeled N-terminally by green fluorescent protein was predominantly distributed within the cytoplasm. Only a minor fraction of α1C,86 was able to incorporate into the plasma membrane, and this may correspond to low current density that is generally observed upon α1C,86 expression (Keppeler et al., 2000b). The membrane-bound fraction of the labeled α1C,86 did not form characteristic clusters.

**Modulation of Sensitivity to DHP**

Free Ca\(^{2+}\) is known to be important for the high-affinity interaction of Ca\(^{2+}\) channels with DHP calcium channel blockers. However, disruption of Ca\(^{2+}\) sensors increased DHP sensitivity of α1C,86 3.5-fold compared with α1C,77 over a wide range of potentials (Soldatov et al., 1997; Zühike et al., 1998). Thus, the modulation of DHP sensitivity observed in α1C,86 occurs in a 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 α1C,105 isoform with a modified transmembrane segment IIS6 (Zühike et al., 1998). Unlike the α1C,86α1C,72 and α1C,105 channels, α1C,70 produced by substitution of exon 22 for 21, showed an altered voltage-dependent inhibition of Ba\(^{2+}\) current by isradipine at very negative potentials (Soldatov et al., 1995). The slope of the IC\(_{50}\) curve at -90 mV was significantly less steep in α1C,70 than in α1C,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 IIS2, 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 α1C but outside of the high-affinity binding site for DHPs. These altered pharmacological properties of the α1C 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 (Abernethy and Schwartz, 1999).

**Truncated Forms and Mutants of α1C**

Screening of brain and fibroblast transcripts has revealed a dominant truncated form of the human α1C calcium channel subunit, which originates from the utilization of an alternative splice acceptor site at the 5’-end of exon 15, conferring 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 IIS6 in as many as 75% of α1C 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 α1C 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 β subunits by the α1C-β interaction site that is retained in the repeat I–II linker.

Structure-functional studies of naturally occurring mutations that affect 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 α1C 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 IIS6, which significantly impaired voltage-gated inactivation (Soldatov et al., 2000). Such a “leaky” mutant may cause Ca\(^{2+}\) overload of the cell and cytotoxicity; however, this remains to be proven.

The remarkable structure-functional diversity of the α1C calcium channel requires further systematic investigation. An interesting approach for exploration of functional links in calcium channel regulation was gained with coexpression of Ca\(^{2+}\) channel isoforms with receptors that have in vivo functional interaction with the Ca\(^{2+}\) channel. For example, coexpression of the α1C,77 channel with the angiotensin type IA receptor in Xenopus oocytes allowed study of regulation of the L-type Ca\(^{2+}\) channel by angiotensin. This regulation was mediated via IP\(_3\)-induced intracellular Ca\(^{2+}\) release and occurred at the molecular motif responsible for the Ca\(^{2+}\)-induced inactivation of the channel (Oz et al., 1998). Use of diverse functional isoforms of Ca\(^{2+}\) channel as biosensors and the measurement of voltage-gated Ca\(^{2+}\) and 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 Ca\(^{2+}\) channel inactivation mechanisms, particularly of its Ca\(^{2+}\)-dependence, 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 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


