Cav1.2, Cell Proliferation, and New Target in Atherosclerosis

Nikolai M. Soldatov
Humgenex, Inc., Kensington, MD 20895, USA

Correspondence should be addressed to Nikolai M. Soldatov; soldatovn.humgenex@verizon.net

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Cav1.2 calcium channels are the principal proteins involved in electrical, mechanical, and/or signaling functions of the cell. Cav1.2 couples membrane depolarization to the transient increase in intracellular Ca$^{2+}$ concentration that is a trigger for muscle contraction and CREB-dependent transcriptional activation. The CACNA1C gene coding for the Cav1.2 pore-forming $\alpha_{1C}$ subunit is subject to extensive alternative splicing. This review is the first attempt to follow the association between cell proliferation, Cav1.2 expression and splice variation, and atherosclerosis. Based on insights into the association between the atherosclerosis-induced molecular remodeling of Cav1.2, proliferation of vascular smooth muscle cells, and CREB-dependent transcriptional signaling, this review will give a perspective outlook for the use of the CACNA1C exon skipping as a new potential gene therapy approach to atherosclerosis.

1. Introduction

It has been long known that Cav1.2 calcium channel blockers inhibit human brain tumor [1], pancreatic cancer [2, 3], breast cancers [4] and small cell lung cancer [5] because they inhibit cell proliferation and DNA synthesis. Correlation between the oncogenic transformation and expression of both Cav1.2 and Cav3 channels was demonstrated in spontaneously immortalized 3T3 fibroblasts [6, 7] suggesting that both types of calcium channels may play a role in cell proliferation. Indeed, studies showed that Cav3 (T-type) calcium channels regulate proliferation, for example, of BC3H1 cells [8], vascular smooth muscle (VSM) cells [9], and glioma, neuroblastoma, and neuroblastoma × glioma hybrid cells [10]. Unlike the majority of cells, including the listed ones, normal human fibroblasts express only Cav1.2 [11]. The pore-forming $\alpha_{1C}$ subunit of this channel was cloned from human fibroblasts and identified as a "short" (exon 1) isoform of the $\alpha_{1C}$-coding gene CACNA1C [12]. A variety of Cav1.2 calcium channel blockers, including dihydropyridines (DHPs) nifedipine and nicardipine, as well as diltiazem and verapamil inhibit cell proliferation and DNA synthesis in fibroblasts [13]. Thus, human fibroblast is an excellent cell type to study the roles of Cav1.2 in proliferation not complicated by expression of other Cav, genetic variants, cell transformation, and/or differentiation.

2. Cav1.2 and Proliferation of Normal Human Fibroblasts

Our earlier studies performed on normal human diploid fibroblasts have revealed a number of important features that point to the plasticity of the Cav1.2 expression in response to cell culture conditions, including cell-cell contact inhibition, presence of mitogens, and second messengers. The expression of Cav1.2 in the plasma membrane was measured using the DHP radioligand binding assay. DHPs bind to Cav1.2 with high affinity in equimolar ratio and are excellent probes for the expression of total (functional and dormant) Cav1.2 in the plasma membrane. The test system was based on the measurement of specific binding of 2,6-dimethyl-3-methoxycarbonyl-5-((2,3-3H$_2$)-n-proxycarbonyl)-4-(2'-difluoromethoxyphenyl)-1,4-dihydropyridine ([H]PMD, NCBI PubChem CID 14267917) [15] to human embryonic diploid fibroblasts grown in Eagle’s medium supplemented with 10% serum. Under standard conditions of incubation (1 h at room temperature in Tris-buffered saline) ([H]PMD interacted with $K_d \approx 3.9$ nM with a single class of DHP receptors that were present at the maximum density ($B_{max}$) of $=1.2$ pmol/10$^6$ cells in a sparse culture of fibroblasts ($=3-5 \times 1,000/cm^2$) [16]. The turnover rate of DHP receptors is approximately exponential with a half-life of $\approx12$ h, as it was
Estimated from the rate of loss of $[^3H]$PMD binding sites in response to the net inhibition of protein synthesis by cycloheximide. With progression to confluent monolayers, the $K_d$ value for $[^3H]$PMD binding did not change, but the $B_{max}$ value decreased $=4$ fold (Figure I(a), compare bars 1 and 2), suggesting that expression of Ca$_{1.2}$ is responsive to the arrest of fibroblasts proliferation by cell-cell contact inhibition.

The involvement of Ca$_{1.2}$ in proliferation of human fibroblasts was further supported by the finding that concentration of DHP receptors, and, respectively, of Ca$_{1.2}$ is strongly affected by mitogens and second messengers. Demonstrating a remarkable plasticity of the Ca$_{1.2}$ expression in normal human fibroblasts (Figure I(b)), serum deprivation induced a 2-fold increase in the density of DHP receptors that reached its maximum after 3-4 days of cultivation in the absence of serum (compare also bars 2 and 6 in Figure I(a)). The elevation of the Ca$_{1.2}$ expression was fully reversible and highly sensitive to serum and other mitogens. An addition of 10% serum reduced the density of DHP receptors to the initial level with almost the same time course (Figure I(b)). Thus, inhibition of cell growth, proliferation, and DNA synthesis in fibroblasts by serum deprivation stimulates expression of Ca$_{1.2}$ with a time course comparable with the DHP receptor turnover rate.

The response of fibroblasts to serum deprivation by boosting Ca$_{1.2}$ expression is essentially identical to that caused by the inhibition of Ca$_{1.2}$ by 1$\mu$M diltiazem (Figure I(a), compare bars 5 and 6), the calcium channel blocker that does not compete with DHPs for binding with the channel. In fact, diltiazem was present in the DHP binding assay medium throughout all experiments to enhance the affinity of the DHP probe to the channel receptor [17]. Thus, it is reasonable to suggest that by boosting the Ca$_{1.2}$ expression, the cell recruits more routes for the Ca$^{2+}$ entry through the plasma membrane to overcome the lack of mitogens in serum deprivation or lack of conducting channels in the presence of diltiazem, both aimed at supporting cell proliferation until cell-cell contact inhibition terminates it and turns the cells into a quiescent state.

If this hypothesis is true, then the addition of DHP-insensitive routes for Ca$^{2+}$ entry through the plasma membrane should eliminate the need in higher level of Ca$_{1.2}$ expression. This observation exactly has been made when the serum-stimulated cells were supplemented with Ca$^{2+}$ ionophore A23187 (Figure I(a), bar 4). The A23187-induced Ca$^{2+}$ entry dramatically reduced the cellular expression of Ca$_{1.2}$. A similar effect was observed with 8Br-cAMP, the plasma membrane-permeable derivative of cAMP, showing that stimulation of the alternative cAMP-dependent cell signaling pathway may reduce the needs in Ca$_{1.2}$ in proliferation of fibroblasts.

Stimulation of Ca$_{1.2}$ expression in fibroblasts, arrested in the quiescent state by serum deprivation, strongly depends on cell proliferation. The measurement of [14C]thymidine incorporation as an assay for DNA synthesis in cells showed

![Figure I: Effect of serum and second messengers on the density of DHP receptors in normal human fibroblasts. (a) On day 2 after the plating of fibroblasts at high (bar 1) or low density (bars 2-6), the growth medium was supplemented with 1mM 8Br-cAMP (bar 3), 1$\mu$M Ca$^{2+}$ ionophore A23187 (bar 4), 1$\mu$M D-cis-diltiazem (bar 5), or growth medium was replaced by serum-free Eagle's medium containing 0.1% BSA (bar 6). After 4 days, the density of DHP receptors was measured at 2nM $[^3H]$PMD specifically bound per 10$^6$ cells of confluent (bar 1, $>2 \times 10^4$ cells/cm$^2$) or nonconfluent monolayers (bars 2-6, 3-5 $\times 10^4$ cells/cm$^2$). Mean of 5-6 measurements ± SEM. *P < 0.05. (b) Effect of serum deprivation on the density of DHP receptors in fibroblasts. On day 2 after plating, sparse cultures of fibroblasts were incubated for up to 4 days in serum-free Eagle's medium supplemented with 0.1% BSA. The horizontal bars indicate when serum-free medium was replaced by standard growth medium containing 10% serum. The time course of changes in the density of DHP binding sites was measured as in (a) at different time points (not shown for simplicity) and compared with that in cells not subjected to serum-deprivation (blue line).]
Recent advances in molecular genetics studies have established that individual mitogens suppressed expression of Ca\(_{\alpha1.2}\) splice variants (coexpressed with \(\beta_1\) and \(\delta\)-1) were compared in Xenopus oocyte expression system [31]. Characteristics of the voltage-dependence and kinetics of inactivation of the barium current through the \(\alpha_{1C,70}\) channel encoded by the CACNA1C transcript lacking exons 22, 31, 41A and 45 were found to be very similar to those recorded with \(\alpha_{1C,77}\) (lacking exons 21, 31, 41A and 45) and \(\alpha_{1C,76}\) (also lacking exon 33) (Figure 3(b)). However, voltage dependence of the DHP inhibition of the current was significantly different in the \(\alpha_{1C,70}\) and \(\alpha_{1C,77}\) channels: the IC\(_{50}\) values for the concentration dependence of the barium current inhibition by (+)-isradipine, almost identical (6.2 and 7.3 nM, resp.) at −40 mV, were significantly different at −90 mV (680 and 79 nM for \(\alpha_{1C,70}\) and \(\alpha_{1C,77}\), resp.). While such a difference in the pharmacological properties of the exon 21- and exon 22-splice variants is deemed unimportant in the case of fibroblasts, it may significantly contribute to the tissue specificity of this major class of calcium channel blockers in cardiac, vascular, and other responsive cells affected by cardiovascular diseases.

4. Ca\(_{\alpha1.2}\) in Atherosclerosis

Atherosclerosis is perhaps the single most deadly disease, leading to about 600,000 deaths annually in the USA, most of these due to the progression of the disease to heart attack or stroke [32]. In spite of significant efforts, the molecular mechanisms of atherosclerosis are not currently well understood, and effective molecular targets for prevention and treatment are not elaborated. Atherosclerosis is an inflammatory process in medium and large size arteries that causes endothelial perturbation and local release of cytokines, as well as dedifferentiation, proliferation, and migration of VSM cells [33]. Arterial VSM cells constitute the media of the artery and play a crucial role in its elasticity and contractility. Migration of VSM cells from the media to the intima of the arterial wall and proliferation of intimal smooth muscle cells are the major early events in the formation of atherosclerotic lesions. Recent advances in molecular genetics studies have
revealed that genetic polymorphisms significantly influence susceptibility to atherosclerotic vascular diseases [34]. However, none of the discovered susceptibility genes was directly implicated for proliferation and migration of VSM cells, one of the major pathophysiological responses to atherosclerosis at the cellular level.

The presence and activity of Ca_{1.2} calcium channels in VSM cells has been established both in patch clamp and molecular cloning experiments [29, 35–38]. Ca_{1.2} calcium channels play a major role in atherosclerosis because they are essential for Ca^{2+} signal transduction in VSM cells. Contraction of VSM cells is triggered by the Ca^{2+} current (I_{Ca}) through Ca_{1.2}, and thus is affected by Ca^{2+} channel blockers. Since the 1990s, it is known that DHPs, particularly, the charged 2-aminoethoxymethyl DHP derivative amlodipine [39], exert a number of vasoprotective effects, including potent antiatherogenic action [40], inhibition of migration of VSM cells [41], and reduction of arterial intimal-medial thickening and plaque formation [42, 43]. Although it is tempting to link this activity in part to pleiotropic effects of

Figure 3: Molecular diversity of Ca_{1.2}. (a) Transmembrane topology of the Ca_{1.2} αIC subunit in schematic diagram. Four regions of homology (I–IV), each composed of 6 transmembrane segments (numbered), are folded around the central pore. The polypeptide sequence is segmented by black bars and sequentially numbered (1–50) according to the CACNA1C genomic map [14]. The segments corresponding to the invariant (black/gray) and alternative (blue) exons are outlined. αC-interaction domain (AID) of a constitutive β-binding is shown in red. In the CaM-binding domain (CBD), apo-CaM and Ca^{2+}-CaM are shared between LA and IQ motifs (red), respectively. (b) Alternative splicing of human fibroblast CACNA1C in the region of exons 20–46. The following splice variants were studied: αIC_{69} (GenBank #z34809, splice variant lacking alternative exons (Δ) 21, 32, 41A, and 45), αIC_{70} (z34810, Δ 22, 31, 41A, and 45), αIC_{71} (z34811, Δ 22, 31, and 45), αIC_{73} (z34812, Δ 22, 31, 33, and 45), αIC_{76} (z34814, Δ 21, 31, 33, 41A, and 45), and αIC_{77} (z34815, Δ 21, 31, 41A, and 45).
calcium channel blockers [44], it is the inhibition of proliferation that essentially underlies it [45]. The density of DHP receptors was found to depend on VSM cell proliferation [46]. Association of Ca_{1,2} with mitogenesis in VSM cells is supported by the findings that DHPs reduced DNA synthesis stimulated by serum and PdGF [47–49], serotonin [50], EGF [51], and H_{2}O_{2} (in mesangial cells) [52]. Expression of Ca_{1,2} in VSM cells was shown to be cell cycle dependent, with the highest calcium current density in G_{1} phase [53]. Whether these changes are reflected in the molecular repertoire of the Ca_{1,2} splice variants is the issue particularly important for the elucidation of new therapeutic targets in diseases leading to pathogenic proliferation of VSM cells, such as atherosclerosis.

Investigation of the α_{1C} alternative splicing in VSM cells [28, 54, 55] revealed the involvement of a number of CACNA1C exons generating impressive diversity of human vascular α_{1C} that includes possibly the VSM-specific splicing of exons 9/9a [35, 55] and exon 34 [28]. To establish an association between the disease and CACNA1C splice variation at the level of cell, we have completed [26] the single-gene profiling of the α_{1C} molecular remodeling in VSM cells of an artery caused by atherosclerosis. The VSM cells were identified in frozen sections of six surgical biopsy samples of femoral and carotid arteries by immunostaining with an antibody against smooth muscle α-actin [56], used as a marker for VSM cells. The α-actin staining correlated with immunostaining by an anti-α_{1C} antibody in serial sections and was reduced in atherosclerotic regions (Figure 4) consistent with dedifferentiation of VSM cells [57, 58]. The reduced expression of α_{1C} at the protein level was corroborated by the quantitative RT-PCR data showing that the relative α_{1C} mRNA level in VSM cells (normalized to 18S RNA) was reduced 3.7 ± 0.9 fold (mean ± SEM) in the atherosclerotic region. Overall, the reduced expression of α_{1C} caused by the locally elaborated cytokines in the atherosclerotic regions of arteries resembles the reduced expression of DHP receptors observed in fibroblasts exposed to mitogens and/or second messengers after serum deprivation (Figure 1).

To find out whether the altered expression of α_{1C} in atherosclerotic VSM cells is accompanied by changes in the CACNA1C alternative splicing pattern, we isolated the immunohistochemically identified VSM cells by laser-capture microdissection from adjacent regions of arteries affected and not affected by atherosclerosis and identified the CACNA1C splice variants by RT-PCR. Our findings revealed an extended repertoire of the exon 21 α_{1C} splice isoforms in nonatherosclerotic VSM cells characterized by a complex splicing pattern of exons 9, 9A, 31–34, and 41A, including the electrophysiologically characterized α_{1C21} (GenBank # z34811), α_{1C23} (z34812), α_{1C25} (AY830711), α_{1C26} (AY830713), and α_{1C27} (AY830712) splice isoforms. However, only the exon 22 isoform of α_{1C} (α_{1C27}) was identified in atherosclerotic VSM cells. Thus, the switch of the CACNA1C alternative splicing from exon 21 to exon 22 (Figure 3(b)) is a molecular signature of the Ca_{1,2} remodeling of VSM cells to a pathophysiologically proliferating state in atherosclerosis. The age, gender, ethnicity, drug exposure, and other co-morbid conditions did not appreciably affect this common pattern of the α_{1C} splice variation in VSM cells in response to atherosclerosis.

Careful electrophysiological analysis exhibited a number of differences in the properties of the “atherosclerotic” α_{1C27} channel as compared to the α_{1C} isoforms in healthy VSM cells. The largest differences were found between the α_{1C27} and α_{1C22} channels (Figure 5). In response to step depolarization applied from the holding potential of −90 mV, both channels generate calcium currents (I_{Ca}) that inactivate with almost identical kinetics (Figure 5(a)). However, we found that I_{Ca} through the α_{1C27} channel recovers from inactivation significantly faster than that in α_{1C22} (Figure 5(b)) and other α_{1C} isoforms present in healthy VSM cells. This finding suggests that alternative splicing in atherosclerosis may affect vascular tone as a result of the increase in the I_{Ca} density in VSM cells. A hyperpolarization shift of the activation curve for the atherosclerotic α_{1C27} channel variant, as compared to Ca_{1,2} in healthy VSM cells (Figure 5(c)), may also result in an increase of calcium entry in VSM cells. However, the overall 3-4-fold reduction in the expression of Ca_{1,2} in atherosclerotic VSM cells may scale down some of the observed electrophysiological changes.

5. CACNA1C Exon 22 as a New Therapeutic Target in Atherosclerosis

Direct DNA sequencing of the crude PCR amplification products indicated that the switch to the exon 22 isoform of vascular α_{1C} was almost complete in atherosclerosis because no distortion of the nucleotide peaks in the region of exon 21/22 was seen when compared to the exon 20 invariant region (Figure 6(a)). Thus, Ca_{1,2} underwent almost quantitative exon 21/22 remodeling in VSM cells of diseased artery regions.

Although the cellular mechanisms leading to the CACNA1C exon 21/22 switch may be very complex, the association with VSM cell proliferation is obvious. Indeed, a similar exon switch was observed in primary human aortic cells in culture after the quiescent nonproliferating cells, containing predominantly exon 21 splice variants, were exposed to serum (Figure 6(b)). Unlike exon 21, exon 22 contains the AvrII restriction site that allows for the assessment of its presence in PCR amplification product of CACNA1C transcripts isolated from the cells. The AvrII-sensitive exon 22 isoform of the α_{1C} transcript was not detected in the quiescent nonproliferating aortic cells (Figure 6(b), lane 2). However, when 5% serum was added to the medium with nonconfluent aortic cells, DNA biosynthesis was activated, while the level of the α_{1C} transcript decreased ≥3 fold, and the presence of the AvrII-sensitive exon 22 isoforms of α_{1C} was easily detected (Figure 6(b), lane 4). The isoform remodeling simulated in aortic primary cells in vitro was not complete as compared to VSM cells in atherosclerotic regions of artery occluded with heavy plaque burden, which were selected for the α_{1C} molecular profiling. However, the cell culture results demonstrate that in a different experimental system, there is an obvious association between proliferation of VSM cells,
downregulation of the CACNA1C expression, and synthesis of the exon 22 α_{1C} isoform.

Recent strategies targeting VSM cells to treat cardiovascular diseases suggest indiscriminate disruption of Ca_{\text{v}1.2} [59–61]. Is it possible to correct the described CACNA1C splice defects induced by atherosclerosis without affecting the transcripts of the gene lacking the "pathogenic" exon 22? Correction of defective genes responsible for disease development is achieved by gene therapy. Usually it requires an insertion into the genome of a normal gene in place of a defective one causing disease. Such technique, however, is poorly controlled. Currently, one of the most promising, cutting-edge therapeutic approaches to correct defects associated with disease-induced expression of abnormal splice variants is antisense-mediated exon skipping. It is based on the use of antisense oligonucleotides targeting specific exons to be removed. The adenovirus-directed α_{1C} exon 22 skipping-induced inhibition of VSM cell proliferation (and, respectively, migration) can be used to rescue VSM cells from remodeling in atherosclerosis. The α_{1C} exon 22-skipping will not alter the open reading frame of the α_{1C} transcript because alternative exons 21 and 22 are of the equal size (60 nt). The modified nonspliceosomal snRNA U7 gene along with its natural promoter and 3' elements, exon 22-antisense
**Figure 5**: Comparison of electrophysiological properties of $I_{Ca}$ through the “atherosclerotic” $\alpha_{1C,77}$ and “normal” $\alpha_{1C,127}$ channels expressed in *Xenopus* oocytes with $\alpha_\delta$-1 subunit and the primary cardiac $\beta_2a$ subunit and measured with $2.5 \text{mM Ca}^{2+}$ as the charge carrier. (a) Representative traces of $I_{Ca}$ evoked by 1s step depolarizations to $+20 \text{mV}$ from $V_h = -90 \text{mV}$ and normalized to the same amplitude. (b) Fractional recovery of $I_{Ca}$ from inactivation. (c) Averaged activation ($G/G_{\text{max}} - V$) curves fit by the Boltzmann function. A 1s test pulses in the range of $-40$ to $+50 \text{mV}$ (10-mV increments) were applied from $V_h = -90 \text{mV}$ with 30-s intervals.

**Figure 6**: (a) Trace diagrams of DNA sequencing of the $\alpha_{1C}$ PCR amplification products of healthy (top row) and diseased VSM cells (bottom row) using an antisense primer composed of nucleotides 2923–2939 (z34815). A boundary with the sequence of invariant exon 20 is marked by a vertical dotted line. (b) Evidence that the AvrII-sensitive exon 22 isoform of $\alpha_{1C}$ is expressed only in proliferating VSM cells. Primary aortic cells were grown to confluent monolayer in 5% serum before serum-deprivation for 5 days (lanes 1 and 2). Then, the cells were replated at low density in 5% serum for 3–4 days (lanes 3 and 4). Total RNA was isolated, and exon 21/22 isoforms were identified by RT-PCR and AvrII restriction analysis (lanes 2 and 4).
sequence and supplemented with Sm ribonucleoprotein-binding sequence, may be incorporated into the adenovirus vector for high efficiency transfer [62]. The cytokine receptors (e.g., PdGF-β receptor) based recognition targeting of viral liposomes or nanoparticles may be especially advantageous in connection with selective gene transfer to VSM cells affected by atherosclerosis, while reducing the probability of the transfection of other cells.

6. Ca$_{1.2}$ and CREB-Dependent Transcriptional Activation

How Ca$_{1.2}$ activity is translated into a proliferation-effected modality is another important question to be asked. Ca$_{1.2}$ calcium channels generate a transient rise in cytosolic Ca$^{2+}$-concentration activated by membrane depolarization. Cellular responses associated with the rise of [Ca$^{2+}$]$_i$, range from sarcomastic contraction to cell growth and proliferation. Cytoplasmic domains of Ca$_{1.2}$ have evolved a fairly intricate CaM-dependent signaling mechanism that provides for the negative feedback inhibition of the calcium current, known as Ca$^{2+}$-dependent inactivation (CDI), which is mediated by different determinants of α$_{1C}$ [25, 63]. Such a mechanism of CDI, resulting in acceleration of $I_{Ca}$ inactivation in response to the rise of intracellular Ca$^{2+}$, was first identified in cardiac Ca$_{1.2}$ [64]. Similar experiments performed on the recombinant Ca$_{1.2}$ also showed that the replacement of extracellular Ca$^{2+}$ by Ba$^{2+}$ eliminates CDI, and the channel inactivates by a slower voltage-dependent mechanism [65]. The very fact that two distantly located determinants, one in the pore region responsible for slow inactivation, and the CaM-binding one in the proximal locus of the C-tail, are independently crucial for CDI indicates that not only their specific molecular structure but also their mutual folding and/or interaction are essential. Experimental evidence show that this interaction reacts dynamically to membrane voltage supporting state-dependent transitions of the channel between resting, open, and inactivated conformations, which are essential for CREB-dependent transcriptional activation [66, 67].

CREB is a transcription factor of general importance in a large variety of cells. CREB phosphorylation promotes the activation of genes and is regulated by protein kinases under control of the major second messengers, cAMP and/or Ca$^{2+}$. Indeed, CREB functions as a "molecular determinant of VSM cells fate" [68]. CREB content depends on proliferation of VSM cells both in situ and in culture. Serum deprivation increased CREB content in VSM cells, while exposure to PdGF decreased it. Consistent with this observation, an overexpression of the constitutively active CREB in VSM cells arrested cell cycle progression.

To investigate the association of Ca$_{1.2}$ with CREB transcriptional activation at the molecular level, we combined patch clamp with fluorescent resonance energy transfer (FRET) microscopy in the live cell. In voltage clamped cells, FRET provided optical measurements under state-dependent conditions showing that the shorter N-terminal tail of α$_{1C}$ (e.g., α$_{1C,77}$) does not rearrange vis-à-vis the plasma membrane in response to voltage gating [25]. In sharp contrast, the C-tail shows voltage-dependent conformational rearrangements, which are much larger in size than that, for example, in the potassium K$_{2,1}$ channel [69]. Measurements of $I_{Ca}$ and corrected FRET between the enhanced yellow (EYFP) and cyan fluorescent proteins (ECFP), genetically attached, respectively, to the N- and C-termini of α$_{1C,77}$ showed no significant effect on voltage-dependence and kinetics of the channel current. However, there was a substantial increase in FRET signal accompanying inactivation of the channel that was fully reversible upon its transition into the resting state in response to hyperpolarization [66]. The plasma-membrane anchoring of the α$_{1C}$ C-tail by the fusion of the pleckstrin homology domain (PH) eliminated CDI but not $I_{Ca}$. Do the voltage-gated conformational rearrangements of the α$_{1C}$ C-tail, and CDI, play a role in Ca$^{2+}$-induced activation or CREB-dependent transcription? To answer this question, we used the test system based on the measurement of interaction between KID domain of CREB and KIX domain of coactivator CREB-binding protein (CBP, Figure 7(A), inset) under voltage-clamp conditions (CBP, Figure 7(A), inset) under voltage-clamp conditions [66]. Ca$^{2+}$-dependent phosphorylation of KID stimulates its binding to KIX, bringing EYFP and ECFP close enough to observe the interaction by FRET. In perforated whole clamped cell, where the integrity of the cytoplasmic content is intact (Figure 7(A)), no activation of CREB transcription (Figure 7(B), panel (a)) and rearrangement of the α$_{1C,77}$ C-tail (panel (b)) was observed when the C-tail was anchored to the plasma membrane. CREB transcriptional activity remained low in spite of a large sustained inward $I_{Ca}$ (panel (d)) and the corresponding increase in [Ca$^{2+}$]$_i$ detected by the fluorescence of Ca$^{2+}$ indicator Fura4 (panel (c)). Release of the α$_{1C,77}$ C-tail by the activation of PIP$_2$ hydrolysis upon activation of M1AchR (Figure 7(C)) at ~90 mV caused significant elevation of [Ca$^{2+}$]$_i$ that also was not utilized by the cell for CREB transcriptional activation (Figure 7(C), panel (a)) until a depolarizing pulse to +20-mV was applied and the released α$_{1C,77}$ C-tail was permitted to rearrange (Figure 7(D)). This experiment provides compelling evidence that neither large inward $I_{Ca}$ nor the subsequent rise in [Ca$^{2+}$]$_i$ lead to CREB transcription activation, unless the conformational rearrangement of the α$_{1C,77}$ subunit C-tail provides the precise targeting of the Ca$^{2+}$ signal transduction (Figure 7(D), scheme) [66].

There is general agreement that CaM binds to LA and IQ domains of the α$_{1C}$ C-tail, and acts as a sensor that conveys CDI (for review, see [63]). The affinity of CaM for both domains depends on [Ca$^{2+}$]$_i$. Our data indicate that CDI and Ca$^{2+}$-signal transduction depend on the voltage-gated mobility of the α$_{1C,77}$ C-tail. It is therefore reasonable to suggest that the LA-domain is a Ca$^{2+}$-sensitive apo-CaM-mediated lock for the mechanism of slow voltage-dependent inactivation of the channel [67]. Apo-CaM associated with LA is able to cross-link it to another, still unidentified apo-CaM binding site in the polypeptide bundle underlying the pore. As the result of this specific localization, apo-CaM/LA "lock" is hidden from the cytoplasmic Ca$^{2+}$ so that, for example, the
intracellular Ca\(^{2+}\) released from the intracellular stores or Ca\(^{2+}\) caging compounds does not accelerate significantly the inactivation of Ca\(_{\alpha1,2}\) [71]. Thus, apo-CaM associated with LA binds predominantly Ca\(^{2+}\) ions permeating through the pore. A Ca\(^{2+}\)-dependent transfer of CaM from LA to the IQ-motif opens the "lock" and initiates a large rearrangement of the C-terminal tail. This in turn facilitates inactivation of the channel. The Ca\(^{2+}\)/CaM complex with the IQ-motif is then transferred by the mobile C-tail to a downstream target of the Ca\(^{2+}\)-signaling cascade (such as CaMKII [72]), where Ca\(^{2+}\) is released as an activating stimulus, while CaM switches back to LA and returns the C-tail to the resting position available for the next cycle of Ca\(^{2+}\)-signal transduction.

Activation of CREB-dependent transcription by the L-type I\(_{\alpha1}\) is mediated through multiple cell signaling pathways. Using FRET probes of CREB activity and 2D wavelet transform analysis, we applied principles of quantitative biology to detail the mechanism of Ca\(^{2+}\)-activated CREB-dependent transcription within localized regions (microdomains) of the nucleus. We reached this goal by applying continuous wavelet analysis in two dimensions with a 2D wavelet as a deconvolution algorithm for FRET microscopy image

Figure 7: Evidence that Ca\(^{2+}\) signal transduction by Ca\(_{\alpha1,2}\) to activate CREB-dependent transcription is mediated by the voltage-gated mobility of the \(\alpha_{1C}\) C-tail carrying the CaM-caged Ca\(^{2+}\) and is not directly associated with the increase of [Ca\(^{2+}\)]. CREB activation was examined under perforated patch conditions in COS1 cells expressing recombinant atherosclerotic \(\alpha_{1C,77}\) channel. (A) Phase-contrast image of the COS1 cell with a shadow of patch pipette. The cell was expressing the \(\alpha_{1C,77}\)-PH/\(\beta_{11}/\alpha_{1,\delta-1}\) channel with the membrane-trapped \(\alpha_{1C,77}\) subunit C-tail, type I muscarinic Ach receptor (to release the anchored C-tail in response to activation by Ach) and EYFP-KID and ECFP-KIX domains, both supplemented with nuclear localization sequences. Inset at the bottom: Ca\(^{2+}\) channel. The Ca\(^{2+}\) released as an activating stimulus, while CaM switches back to LA and returns the C-tail to the resting position available for the next cycle of Ca\(^{2+}\)-signal transduction.
Figure 8: Microdomain analysis on the additive effect of $I_{Ca}$ and cAMP on CREB signaling in COS1 cells expressing the recombinant “atherosclerotic” $\alpha_{1C,77}$ channel. (A) FRET signal within the nucleus during selected time points during $I_{Ca}$ stimulation and cAMP application. Outlined are four types of signaling microdomains identified using 2D Mexican hat wavelet. Red circles represent stable microdomains that persist through both $I_{Ca}$ and cAMP application. White and yellow circles show microdomains stable activated by cAMP and $I_{Ca}$, respectively. Green circles represent transient microdomains of CREB signaling activation. Axes show pixel numbers. (B) Typical appearances of the four types of CREB signaling microdomains activated by $I_{Ca}$ and/or cAMP application and recorded in their maximal development in relation to the time of applied stimuli. C: control recorded before stimulation. Color bars in (A) and (B) represent FRET values normalized to the maximum.

analysis [73]. Continuous wavelet analysis is a mathematical technique that allows us to analyze a signal over several different frequencies across the entire signal [73, 74]. It is especially useful for finding heterogeneity in a signal because it can easily find where the pattern (i.e., frequency) of a signal changes. In these experiments, we, for the first time, obtained evidence of CREB signaling microdomains within the nucleus that respond differentially to $I_{Ca}$ stimulation and cAMP (Figure 8(A)). Results of the study revealed that CREB-dependent transcriptional signaling occurs in discrete signaling microdomains underlying the architecture of nuclear signaling. Continuous activation of CREB-dependent transcriptional signaling by cAMP and Ca$^{2+}$ resulted in a gradual increase of the number of microdomains. Four different categories of cAMP and Ca$^{2+}$-induced CREB signaling microdomains were characterized in COS1 cells expressing recombinant Ca$_{1.2}$ with the “atherosclerotic” $\alpha_{1C,77}$ splice variant (Figure 8). In up to 65% of the microdomains, transcription was activated in additive manner by cAMP and Ca$^{2+}$. Approximately 15% of signaling domains were activated only by $I_{Ca}$ and 5% of domains were activated only by cAMP. Finally, 15% of the domains were transient, and activated by both cAMP and $I_{Ca}$ (Figure 8(B)) [75].

A similar spatiotemporal organization of CREB-dependent signaling was observed in spontaneously beating neonatal rat cardiomyocytes. Although COS1 cells that were used in our experiments shown in Figures 7 and 8 are naturally deprived of Ca$_{1.2}$ [76], they inherited the ability to replicate the Ca$_{1.2}$-dependent activation of CREB signaling with the exogenous recombinant channel. Thus, this experimental approach fits the task, which, in my opinion, is the most important unresolved issue for the coupling of Ca$_{1.2}$ to CREB signaling: does the splice variation of $\alpha_{1C}$ affect the spatiotemporal organization of CREB-dependent signaling.
in a way that may affect cell proliferation and other crucial function?

7. Conclusions

Association of Ca\(_{1.2}\) with regulation of transcription, cell proliferation, and its pathophysiology, as in the case of atherosclerosis, requires detailed investigation of the roles of the naturally occurring \(a_C\) splice variants. It will limit the traditionally intuitive approach to Ca\(_{1.2}\) in physiology and help to define new principle approaches to the treatment of various Ca\(_{1.2}\) channelopathy-related dysfunctions, above all cardiovascular diseases. Humgenex Inc. provides consulting and logistic support on a broad range of issues reported in this review.

Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| CaM | Calmodulin |
| CBD | Calmodulin-binding domain |
| CDI | Ca\(^{2+}\)-dependent inactivation |
| DHP | Dihydropyridine |
| ECFP | Enhanced cyan fluorescent protein |
| EYFP | Enhanced yellow fluorescent protein |
| FALL | Fluorophore-assisted light inactivation |
| FRET | Fluorescent resonance energy transfer |
| \(I_{Ca}\) | Current calcium |
| PH | Pleckstrin homology domain |
| VSM | Vascular smooth muscle |

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