Published in final edited form as:
*Cell Calcium*. 2011 May ; 49(5): 306–313. doi:10.1016/j.ceca.2011.01.001.

# L-type Calcium Channel Auto-Regulation of Transcription

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## Abstract

L-type calcium channels (LTCC) impact the function of nearly all excitable cells. The classical LTCC function is to mediate trans-sarcolemmal $Ca^{2+}$ flux. This review focuses on the contribution of a mobile segment of the LTCC that regulates ion channel function, and also serves as a regulator of transcription in the nucleus. Specifically we highlight recent work demonstrating an auto-feedback regulatory pathway whereby the LTCC transcription factor regulates the LTCC. Also discussed is acute and long-term regulation of function by the LTCC-transcription regulator.

## Introduction

L-type calcium channels (LTCC) couple membrane depolarization to cytosolic calcium entry. In turn, calcium bridges excitation to contraction in cardiac myocytes. Cytosolic $Ca^{2+}$-entry via LTCC stimulates a larger release of $Ca^{2+}$ from the sarcoplasmic reticulum (SR) via ryanodine receptors ($RyR_2$). Details of this process are the topic of excellent reviews [1,2]. A second, less-appreciated contribution of $Ca^{2+}$ in cardiac myocytes is to couple excitation to transcription. The goal of this review is to introduce a relatively new role for cardiac L-type calcium channels (LTCC) – a direct signal transduction pathway linking LTCC function to transcriptional regulation. The focus of this review is mainly on cardiac LTCC, but there are broader implications given that LTCC are expressed across a wide-range of tissue types [3].

The heart adapts to changing demands by matching output. This occurs on a broad range of time scales from acute beat-to-beat changes to long-term growth related changes. Acute sympathetic nervous stimulation causes a positive inotropism on a relatively rapid time scale. On a considerably longer time scale the growth of the heart during maturation is matched to its functional load[4]. Similarly, exercise or pregnancy stimulates heart growth[5] resulting in a physiological and reversible hypertrophy. Numerous signaling cascades are implicated in the regulation of heart growth including, but not limited to $Ca^{2+}$-regulated processes[6]. Heart growth is defined here as a change in heart size, principally cardiomyocyte cell size. The detail of size alterations depend on the specific stimuli[7]. Pathological stimuli also promote heart growth, though the signaling pathways are distinct from growth stimulated by physiological cues[8]. Central to this review, cytosolic $Ca^{2+}$ is a key contributor to a number of signaling systems. $Ca^{2+}$-activated calcineurin (CaN), and calcium-calmodulin dependent kinase (CaMKII) are two examples of $Ca^{2+}$-effectors in



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cardiomyocyte adaptive signaling. Alterations in cytosolic $Ca^{2+}$-entry are upstream of these signaling cascades, and thus represent a potentially sensitive target for regulation of this diverse signaling. In this review we summarize findings that are consistent with LTCC sensing changes in $Ca^{2+}$-entry, and then coupling such changes to reflexive adjustments in LTCC expression via a mobile segment derived from the LTCC.

## 1. LTCC structure

LTCC exists as part of a multi-protein complex in cardiomyocytes[9]. The main pore-forming subunit of the LTCC in the myocardium is $Ca_V1.2$. $Ca_V1.2$ is also expressed in vascular smooth muscle, pancreatic β-cells, neurons, and developing skeletal muscle. Closely related $Ca_V1.1$ is expressed in mature skeletal muscle. $Ca_V1.3$ is expressed in atrial myocytes, neurons, and chromaffin cells. $Ca_V1.4$ channels, largely expressed in retina complete the $Ca_V1$ family. This review focuses mainly on the $Ca_V1.2$ channel. Additional core components that are shared among a variety of cell types include a β-subunit, α2δ-subunit, and in some tissues a γ-subunit, including possibly the heart[10].

The $Ca_V1.2$ channel contains four homologous repeating units joined by cytosolic linkers. Each homologous repeat consists of six α-helical transmembrane segments, a β-sheet pore region, and a voltage sensor comprised of the four S4 transmembrane segments (reviewed by[11]). An amino-terminal cytosolic domain is immediately upstream of homologous repeat I, and a cytosolic domain is immediately downstream of homologous repeat IV, transmembrane segment 6 (IVS6) forming the carboxyl-terminus of $Ca_V1.2$.

The $Ca_V1.2$ carboxyl-terminus extends >600 amino acids from the end of IVS6. There are some discrepancies in size depending on splice variants, species, and tissue-specific expression (see below). The $Ca_V1.2$ carboxyl-terminus consists of two broad regions demarcated by a consensus calpain cleavage site[12]. Following the nomenclature presented by the Catterall lab, the upstream carboxyl-terminus is the proximal C-terminus (PCT), and the carboxyl end is the distal C-terminus (DCT; Figure 1). The PCT contains an IQ domain that serves as a calmodulin (CaM) interaction site [13,14]. Multiple CaM interact with and modulate $Ca_V1.2$ function [15,16] consistent with additional CaM binding sites on PCT [14,17,18]. Moreover, calmodulin kinase II (CaMKII) and the PCT IQ domain facilitates $I_{Ca,L}$[19,20]. PCT also mediates interactions with RGK proteins[21]. A decade ago Bertil Hille in his classical text book noted that $Ca_V1.n$ and $Ca_V2.n$ channels have 7 known interacting proteins[11]. A recent quantitative proteomic study of $Ca_V2$ channels discovered ~200 candidate interacting proteins [22]. It is expected that a similar number of candidate interacting proteins would be found for $Ca_V1$-family channels. Thus, the $Ca_V1.2$ channel is a hub of protein-protein interactions.

A critical protein-protein interaction central to this review is the $Ca_V1.2$ – DCT interaction. The identification of $Ca_V1.2$ truncation at a consensus calpain substrate site [12,23,24], coupled with the presence of ~37kD protein recognized by a DCT-antibody [25] suggests that DCT is generated by proteolytic cleavage of the full-length $Ca_V1.2$ protein. A requirement for $Ca^{2+}$ and calpain activity for PCT cleavage is inferred from sequence data – as yet there is no direct evidence for either a $Ca^{2+}$ or calpain requirement for DCT liberation.

## 2. L-type Calcium Channel (*cacna1c*) Promoter

$Ca_V1.2$ is encoded by the *cacna1c* gene which is located on chromosome 6 in mouse and 12 in human. By all accounts it is a very complex locus. Databases list as many as 35 isoforms. In human there are 7 predicted alternative promoters with variations in the mRNA including 5′ and 3′ truncations[26]. $Ca_V1.2$ expression has been shown to be regulated by β–adrenergic stimulation[27,28], α adrenergic stimulation[27-29], androgens[30,31], elevated

blood pressure[32], atrial tachycardia[33], endothelin via mitochondrial signaling[34], inflammation[35], and calcium entry through the channel[25,36].

Much of the work examining the *cacna1c* promoter has been done in rat. Initial work demonstrated an approximately 2KB 5′ flanking region as a member of the TATA-less class of core promoters [37]. In this work binding sites for transcription factors such as NKX2.5, Mef2c, AP-1, a cAMP response element, and hormone binding sites were identified. In addition, this work demonstrated the tissue specificity of this promoter as well as identified a region critical for promoter activity, a minimal promoter.

The *cacna1c* gene in human and rat has two confirmed alternative promoters [38,39] that result in differential initial exon usage[40]; reviewed by[41] . The distinct promoters drive the expression of two isoforms of $Ca_V1.2$, one predominates in cardiac tissue, and the other expressed in vasculature and other muscle tissues. The majority of the $Ca_V1.2$ in human cardiac tissue expresses a longer first exon[42]. Greater than 90% of human DCT appears as a single expressed set of exons [43]. Nonetheless, down-stream splicing variations have been noted. To date only a limited number of exon-usage patterns have been attributed with specific functional consequences. These include an exon8/8a variant that contributes to DHP sensitivity[44]. Carboxyl-terminus differential exon usage includes, in PCT an alternative 57 nucleotides following exon 40, and, in DCT a mutually exclusive exon45 versus 45* (Figure 1B). Differential exon usage in the carboxyl terminus influences L-type $Ca^{2+}$ current ($I_{Ca,L}$). The shorter the DCT becomes, the greater the current amplitude. Exons 41-42 encode the distal end of the PCT, and influences voltage- and $Ca^{2+}$-dependent inactivation [45-47].

Hormones, specifically testosterone, have been shown to alter the expression of $Ca_V1.2$ mRNA. Experiments examining coronary tissue from swine demonstrated enhanced expression of $Ca_V1.2$ in the male population *in vivo*, with an increased expression of $Ca_V1.2$ in response to testosterone *in vitro*. This *in vitro* response was blocked by an androgen antagonist suggesting testosterone may be a regulator of expression through interaction with hormone binding sites in the $Ca_V1.2$ promoter[48].

More recent work in HL-1 cells showed increased expression of $Ca_V1.2$ mRNA, protein and current in response to Angiotensin II treatment (AngII) [49]. Moreover, AngII increased $Ca_V1.2$ promoter activity [49]. A combination of mutation analysis and truncation of the promoter suggested that the upstream cAMP response element (cre) was the ANG II interaction site [49].

## 3. DCT in the nucleus regulates transcription

LTCC couples membrane depolarization to gene expression [50]. Several mechanisms have been proposed to explain excitation-transcription coupling: 1) signaling by $Ca^{2+}$ secondary to LTCC-mediated cytosolic $Ca^{2+}$-entry; 2) signaling by CaM / CaMKII; and 3) signaling by DCT.

1) LTCC activity is linked to transcriptional regulation of a variety of genes such as c-fos in hypoxia[51], RhoA/Rok, myocardin, and SRF pathways in smooth muscle[52], and in cardiac memory[53]. Cardiac memory describes electrical pacing induced ionic remodeling, leading to changes in action potential duration[54]. The transcription factor CREB (cAMP-responsive element binding protein) is decreased during cardiac memory induction. In contrast, long-term potentiation in neurons is associated with increased CREB. Regardless, in both cardiac memory and LTP, pharmacological blockade of LTCC prevents stimulus-transcriptional signaling (reviewed by [50]). The details relating nuclear signaling to LTCC Ca-entry are less clear. Ventricular pacing induced ionic remodeling requires CaMKII and NFAT activity [55]. Though this signaling requires $I_{Ca,L}$, alternative mechanisms for NFAT

activation exist, including direct activation by IP3[56]. In this vein, agonist stimulated excitation-transcription coupling in cardiac myocytes can involve $Ca^{2+}$-CaMKII- signaling that is apparently independent of beat to beat Ca-signaling [57]. These results are consistent with the notion that micro-domains of intracellular $Ca^{2+}$ dictate downstream responses. Micro-domains of intracellular $Ca^{2+}$ can arise from heterogeneous distributions of LTCC complex components, or temporal and spatial heterogeneity in local $Ca^{2+}$. Restricted populations of LTCC show $I_{Ca,L}$ modulation due to membrane sub-domains containing appropriate signaling complexes[58,59]. By extension, it is plausible to speculate that heterogeneous LTCC complexes, and /or specialized sub-domain localization of LTCC are restricted for excitation-transcription coupling. A mutually exclusive hypothesis is that heterogeneous $Ca^{2+}$ sub-domains form discrete upstream signals for transcriptional regulation. Cardiac myocytes show well-established temporal and spatial cytosolic $Ca^{2+}$ heterogeneities. Temporally, $Ca^{2+}$rises >10-fold from diastole to systole, and spatially the cleft between T-tubules and SR forms a restricted space. Increasing LTCC activity in cardiac myocytes is not necessarily sufficient to elicit transcriptional responses manifested as cardiac hypertrophy [60,61]. This is consistent with the notion that peak (systolic) cleft $Ca^{2+}$ is saturated at baseline. This raises the idea that diastolic $Ca^{2+}$ is an important determinant for myocardial transcriptional signaling. To date, interrogation of cleft diastolic $Ca^{2+}$ has been hampered by sufficient spatial and temporal resolution.

2) Additional interest in $I_{Ca,L}$ CREB signaling stems from the observation that CREB, acting on a cre element on the $Ca_V1.2$ promoter transcriptionally regulates $Ca_V1.2$[49]. In neurons, LTCC function has been implicated in CREB signaling [62-65]. LTCC activity stimulates nuclear localization of phosphorylated CREB (pCREB) via CaM/CaMKII signaling [66]. LTCC-induced activation of CaMKII parallels CREB signaling leading to the conclusion that CaMKII localized to LTCC PCT domains may provide a link from LTCC to nucleus. DCT may also be involved in transducing LTCC – CREB signaling. Anchoring recombinant $Ca_V1.2$ DCT inhibits CREB-dependent transcription in heterologous expression systems[67].

3) Direct DCT transcriptional signaling. A mutually exclusive alternative signal transduction mechanism is that DCT links LTCC activity to transcriptional signaling. Our first clue that the DCT was more than a traditional ion channel regulator was the observation that a substantial fraction of DCT localizes to the nucleus of cardiomyocytes (photo Figure 2). Dolmetsch's laboratory reported DCT nuclear localization in neurons from the brain, and over-expressed in HEK 293T cells[68]. In neurons DCT regulates transcription of a variety of genes including up-regulation of connexin31.1 and down-regulation of NaCa-exchanger 1 [68]. In addition, DCT interacts with nuclear proteins, and stimulates neurite outgrowth[68]. Thus DCT function is multi-faceted. At the cell membrane DCT regulates LTCC current, and in the nucleus DCT is a regulator of transcription.

On general terms homeostatic feedback loops require a sensor, a signal transducer or transfer component, and a resulting output signal. Pharmacological and genetic chronic blockade of $I_{Ca,L}$ resulted in up-regulation of $Ca_V1.2$ protein[69,70]. This suggested the intriguing possibility that LTCC serve as sensor, and via unknown transfer component, was also the target of its own function. There are numerous signaling cascades activated by LTCC $Ca^{2+}$-entry each of which has numerous intermediaries. DCT, by contrast, represents a privileged pathway for communication between LTCC function and transcription. One requirement for DCT homeostatic auto-regulation of LTCC (including DCT) is that DCT interacts with the $Ca_V1.2$ promoter. Chromatin immunoprecipitation assays established DCT – $Ca_V1.2$ promoter interaction with elements containing NKX2.5/MEF, C/EBP, and CRM1 sites, but not with a cre-containing element [25]. Moreover, nuclear localized DCT restricted $Ca_V1.2$ transcription, $Ca_V1.2$ mRNA, $Ca_V1.2$ protein, and LTCC current [25]. This raises

the possibility that dependent on sub-cellular localization DCT can function as part of a sensor, a transfer element, and an effector mechanism in LTCC regulation (Figure 3). Hence, we use the terminology 'LTCC auto-regulation.'

## 4. DCT signaling of cardiac myocyte growth

There is overwhelming evidence that LTCC pharmacological blockade reduces cardiac mass in patients. Animal studies suggest that LTCC pharmacological blockade can be mediated by direct myocardial actions, rather than activity secondary to relief of hypertension. In mice LTCC-mediated $Ca^{2+}$ entry is a proximal signal for cardiac hypertrophy[71], and subpressor doses of nifedipine inhibit cardiac hypertrophy induced experimentally by pressure overload via aortic constriction[72]. LTCC blockade also inhibits cardiac hypertrophic signaling *in vitro* [73]. However, the linkage between LTCC signaling and hypertrophic growth is not entirely clear. Here we focus on potential contributions by DCT. Exogenous over-expressed DCT inhibits fetal bovine serum induced cardiac myocyte hypertrophy [25]. Atrial natriuretic factor (ANF) is marker for pathological cardiac hypertrophy (reviewed by [5]), and DCT over-expression also blocks serum-induced ANF expression. This is consistent with DCT interactions with multiple genes in cardiac myocytes as previously noted for neurons[68]. Closer examination of DCT repression of $Ca_V1.2$ transcription revealed a potentially complex mechanism of action (summarized in Figure 4). Deletion of the cre element from the $Ca_V1.2$ promoter reduces promoter activity [37,38,49], and mutation of the cre interaction site eliminates angiotensin II regulation of $Ca_V1.2$ expression [49]. DCT does not apparently directly interact with the upstream cre element, but the cre element interacting proteins may influence DCT regulation of $Ca_V1.2$ promoter activity. In absence of growth factor, DCT inhibition of $Ca_V1.2$ promoter activity is significantly blunted. Thus, DCT may contribute to Ca homeostasis by governing $Ca_V1.2$ increases of expression.

For DCT to function as a transcriptional regulator it must be present in the nucleus. DCT is a predicted 37-40 kDa protein and thus requires regulated cellular localization rather than simple passive diffusion. To date, only two publications document DCT sub-cellular localization [25,68]. Exposure of neurons to a depolarizing, high-$K^+$ bath solution induces relatively less nuclear localization, and this effect is reversed by LTCC blockade or chelation of bath $Ca^{2+}$[68]. In cardiac myocytes, serum promotes nuclear localization [25]. Thus, the simplest explanation for serum inhibition of $Ca_V1.2$ expression is that serum-signaling promotes nuclear localization (Figure 4), or perhaps inhibits nuclear exclusion. DCT is a repressor of $Ca_V1.2$ expression, therefore, more nuclear DCT results in less $Ca_V1.2$ transcription. These ideas require further examination.

## 5. LTCC, DCT, and acute feedback regulation by $Ca^{2+}$

The unambiguous contribution of LTCC to function is to permit cytosolic $Ca^{2+}$-entry. Depolarization opens LTCC, $Ca^{2+}$-entry occurs, and LTCC inactivation commences. Two major mechanisms limit $Ca^{2+}$-entry: voltage-dependent inactivation (VDI), and $Ca^{2+}$-dependent inactivation (CDI)[74]. VDI may dominate in basal state, whereas CDI becomes a dominate mechanism following β-adrenergic stimulation[75]. CDI occurs when Ca permeating via the LTCC interacts with CaM that is pre-bound to the LTCC. Mutagenesis, functional, and structural studies show that the $Ca_V1.2$ PCT region is a major contributor to CDI. $Ca_V1.2$ truncation mutants lacking DCT continue to exhibit CDI [76,77]; however, immobilization of DCT inhibits CDI [78]. Thus, DCT is not required, but contributes to $I_{Ca,L}$ kinetics – including local feedback signaling by permeating $Ca^{2+}$.

## 6. Modulation of LTCC Involves DCT

$I_{Ca,L}$ via LTCC triggers a greater ryanodine receptor-based $Ca^{2+}$ release in cardiomyocytes. Thus subtle changes in LTCC function lead to greater downstream changes. Sympathetic stimulation of cardiomyocytes causes activation of the β-adrenergic signaling axis, ultimately increasing protein kinase A (PKA) activity. LTCC are a key substrate for β-adrenergic activated protein kinase A. Thus, a critical element of understanding the β-adrenergic signaling axis is a more detailed understanding of LTCC modulation. Investigations aimed at elucidating PKA-phosphorylation-substrate sites on $Ca_V1.2$ have been an area of intensive examination yet remain unsettled in some important respects. Early studies showed that a PKA consensus site on DCT, Ser1928 (numbering using the rabbit ortholog) is phosphorylated by PKA following β-adrenergic stimulation [79-81]; however, follow-up studies convincingly argued against an $I_{Ca,L}$-modulation effect resulting from DCT Ser1928 phosphorylation. Recombinant channels expressed in non-excitable cells do not reconstitute the β-adrenergic – LTCC signaling axis. To overcome this limitation, a clever approach was designed to assess $Ca_V1.2$ point mutations in cardiomyocytes. The nifedipine-binding site was previously localized to domain III transmembrane segment 5 (IIIS5) and IVS6 [82]. Point mutations in these domains rendered $Ca_V1.2$ fully functional but resistant to dihydropyridines (DHP) such as nifedipine. Thus, DHP-resistant channels over-expressed in cardiomyocytes could be distinguished from endogenous LTCC simply by recording $I_{Ca,L}$ in the presence of a dose of DHP to provide maximal native $I_{Ca,L}$ blockade. A DCT point mutation at Ser1928 to Ala on this DHP-resistant channel over-expressed in cardiomyocytes resulted in channel modulation in response to β-adrenergic stimulation[83]. Similarly, a genetic-engineered mouse carrying a knock-in of a Ser1928-CaV1.2 channel retained β-adrenergic modulation of $I_{Ca,L}$[84]. Truncation of DCT at position 1905 significantly blunted modulation[83]. Thus, DCT is necessary for the sympathetic stimulation induced increase of $I_{Ca,L}$. Even though DCT is phosphorylated by PKA, apparently phosphorylation of DCT is not required for channel modulation.

More recently, Catterall's laboratory reported a comprehensive series of recombinant channel studies. PKA modulation of $I_{Ca,L}$ can be reconstituted with recombinant channels with the co-expression of A-kinase anchoring protein (AKAP) along with LTCC subunits[85]. Site directed mutagenesis experiments established that Ser1700 and Ser1704 on the proximal CT (PCT) are substrates for AKAP-localized PKA, and that these PCT phosphorylation-substrates are required for $I_{Ca,L}$ modulation by PKA[86]. Although DCT was required for modulation, DCT phosphorylation was not necessary. Therefore, DCT contributes to $I_{Ca,L}$ modulation, but the functional consequence of DCT phosphorylation is unknown. It should be noted here that DCT is also a substrate for PKC[10], and functional consequences require further exploration.

## 7. Perspective

LTCC $Ca^{2+}$-entry regulates numerous effector pathways that ultimately regulate transcription. In turn, LTCC are regulated by a variety of physiological and pathophysiological stimuli. DCT represents a possible privileged signaling pathway between functioning LTCC and the nucleus. A major question that needs attention is the relative importance of DCT among LTCC-dependent signaling cascades. Further complexity, or perhaps opportunities, for signal transduction crosstalk may arise from the known interactions among DCT – AKAP – CaN – and LTCC-PCT domains.

## Acknowledgments

We are grateful for the many insightful discussions with Dr Douglas Andres. This work was supported by NIH HL074091 (JS), and an NIH Interdisciplinary Cardiovascular Sciences Training Grant HL-072734 (SMC).

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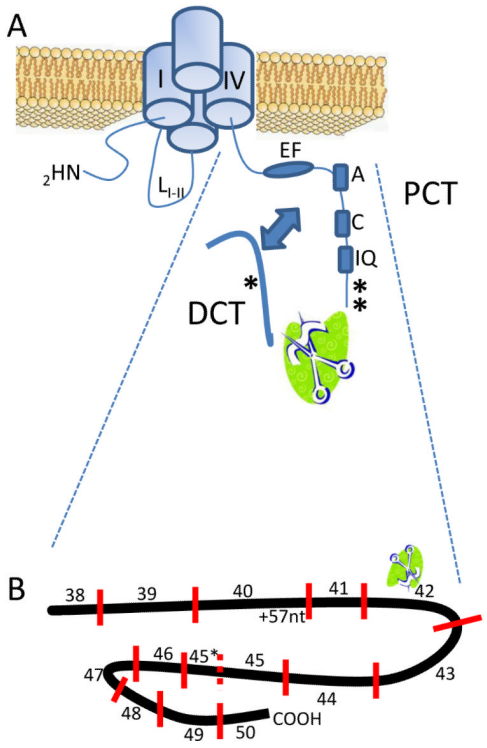


**Figure 1.**

A) Organization of cytosolic domains of the L-type calcium channel. The carboxyl-terminal domain is cleaved in approximately ½ resulting in a mobile distal C-terminus (DCT) separate from proximal C-terminus (PCT) of the pore-forming domains of channel. The arrow represents akap (ref) aiding dCT – PCT interaction. Asterisks (*) show phosphorylation sites.

B) Relationship of exon usage to PCT and DCT. The consensus calpain cleavage site is encoded within exon42.

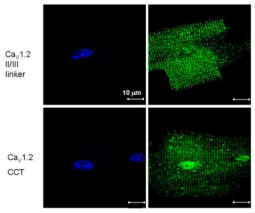


**Figure 2.**

$Ca_V1.2$, upstream of DCT is localized in a T-tubule restricted pattern whereas DCT also localizes to the nucleus in adult mouse ventricular myocytes. Confocal micrographs: Left panels: DAPI; right panels – antibody staining. Negative controls of no primary antibody yielded no detectable fluorescence.

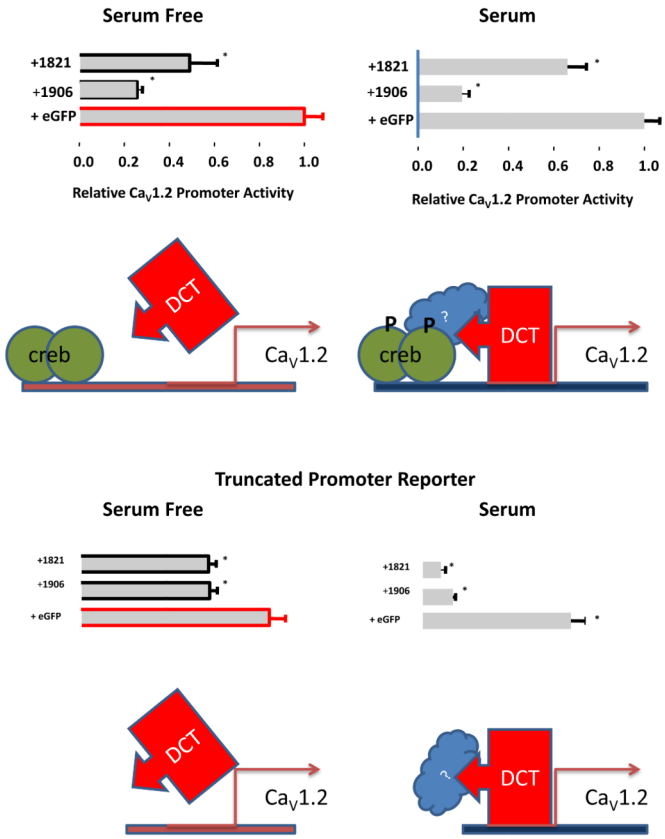


**Figure 3.**
Schematic diagram of DCT - CaV1.2 promoter regulation.

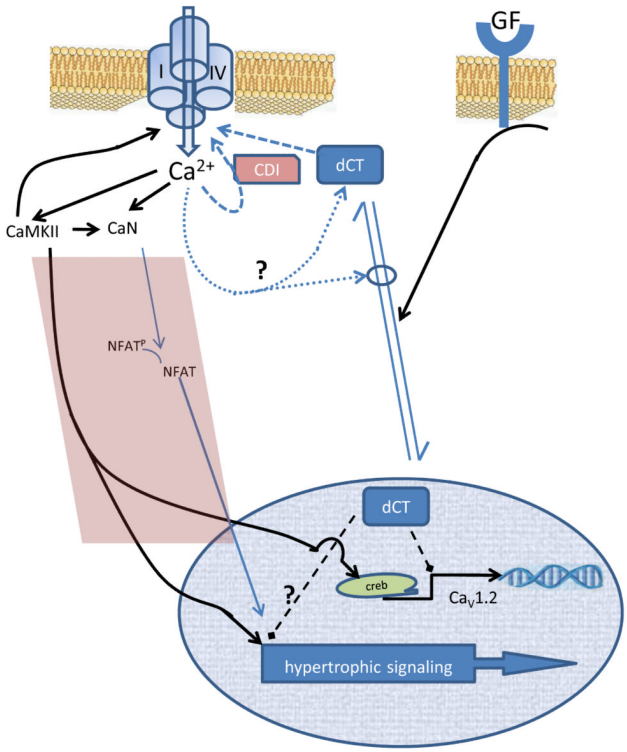


**Figure 4.**

The LTCC - dCT signaling axis. DCT confers signaling in distinct domains of the cell (right side of diagram). In the vicinity of the channel DCT inhibits LTCC current. $Ca^{2+}$-dependent inactivation is the major modulator of $I_{Ca,L}$ whereby local LTCC Ca-entry leads to $Ca^{2+}$-CaM interaction on the PCT driving channel closure/inactivation. In contrast, DCT inhibits LTCC $Ba^{2+}$ current suggesting $Ca^{2+}$-independent current blockade. In the nucleus DCT regulates multiple genes including an interaction with the $Ca_V1.2$ promoter resulting in inhibition of transcription. In neurons, elevated cytosolic $Ca^{2+}$ favors increased cytosolic / nuclear localization. Though speculative, this suggests a negative feedback loop whereby excessive $I_{Ca,L}$ is countered by additional DCT to provide additional $I_{Ca,L}$ blockade. The left side of the diagram (shaded area) depicts feed-forward loops. Increased $I_{Ca,L}$ can increase CaMKII and directly or indirectly increase calcineurin (CaN). CaMKII and CaN may lead to increased hypertrophic signaling. The caveat, however, is that it is unclear whether specific sub-domains of $Ca^{2+}$ are required for hypertrophic signaling. Within the nucleus DCT may also interfere with hypertrophic signaling; although, the effect of dCT on hypertrophy may be secondary to DCT effects on $Ca^{2+}$ signaling. The right side of the diagram represents data supporting growth factors increasing nuclear localization of DCT.