Cell

The C Terminus of the L-Type Voltage-Gated Calcium Channel $Ca_V1.2$ Encodes a Transcription Factor

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DOI 10.1016/j.cell.2006.10.017

SUMMARY

Voltage-gated calcium channels play a central role in regulating the electrical and biochemical properties of neurons and muscle cells. One of the ways in which calcium channels regulate long-lasting neuronal properties is by activating signaling pathways that control gene expression, but the mechanisms that link calcium channels to the nucleus are not well understood. We report that a C-terminal fragment of $Ca_V1.2$, an L-type voltage-gated calcium channel (LTC), translocates to the nucleus and regulates transcription. We show that this *c*alcium *c*hannel *a*ssociated *t*ranscription regulator (CCAT) binds to a nuclear protein, associates with an endogenous promoter, and regulates the expression of a wide variety of endogenous genes important for neuronal signaling and excitability. The nuclear localization of CCAT is regulated both developmentally and by changes in intracellular calcium. These findings provide evidence that voltage-gated calcium channels can directly activate transcription and suggest a mechanism linking voltage-gated channels to the function and differentiation of excitable cells.

INTRODUCTION

Changes in intracellular calcium regulate many cellular events, including synaptic transmission, cell division, survival, and differentiation. Voltage-gated calcium channels are an important route of calcium entry and are essential for converting electrical activity into biochemical events in excitable cells (Catterall et al., 2005). Among the ten different types of voltage gated calcium channels, L-type channels (LTC), encoded by the $Ca_V1.2$ and $Ca_V1.3$ pore-forming subunits, are particularly effective at inducing changes in gene expression that underlie plasticity and adaptive neuronal responses (Bading et al., 1993). Calcium influx through LTCs activates transcription factors such as CREB, MEF, and NFAT (Graef et al., 1999; Mao et al., 1999; Sheng et al., 1990) that lead to the expression of genes such as c-*fos* and *BDNF* (Morgan and Curran, 1986; Murphy et al., 1991; Zafra et al., 1990). Two mechanisms link LTCs, particularly $Ca_V1.2$, to the activation of transcription factors such as CREB. Calcium entering through the channels can diffuse to the nucleus and activate nuclear calcium-dependent enzymes, such as CaMKIV, that regulate the activity of transcription factors and coregulators (Hardingham et al., 2001). In addition, calcium entering cells through LTCs can activate calcium-dependent signaling proteins around the mouth of the channel, which propagate the signal to the nucleus (Deisseroth et al., 1998; Dolmetsch et al., 2001).

In this study we have identified a new mechanism by which calcium channels control gene expression. We report that neurons produce a C-terminal fragment of $Ca_V1.2$ that can regulate transcription and that we call *c*alcium *c*hannel *a*ssociated *t*ranscriptional regulator or CCAT. CCAT is located in the nucleus of many neurons in the developing and adult brain, and its production and nuclear localization are regulated developmentally. In addition, calcium influx through LTCs and NMDA receptors causes CCAT export from the nucleus. In the nucleus, CCAT interacts with the transcriptional regulator p54(nrb)/NonO and can activate transcription of both reporter and endogenous genes. Using microarrays and real-time PCR, we show that CCAT affects the transcription of many neuronal genes, including a gap junction, an NMDA-receptor subunit, and the sodium-calcium exchanger. CCAT binds to the enhancer of the *Connexin 31.1* gene (*Cx31.1*) and directly regulates both the expression of a *Cx31.1* reporter gene and the expression of the endogenous gene. Finally, we show that CCAT expression can cause an increase in neurite extension in primary neurons. This is the first example of a calcium channel having a dual function as an ion pore and a transcription factor.

RESULTS

CCAT Is Found in the Nucleus of Neurons in the Brain

Experiments in neurons and cardiac myocytes have suggested that the C terminus of $Ca_V1.2$ is proteolytically cleaved, yielding a truncated channel and a cytoplasmic C-terminal fragment (De Jongh et al., 1994; Gerhardstein et al., 2000). To investigate the function of the C-terminal fragment, we developed an antibody to a 14-amino acid peptide in the C terminus of $Ca_V1.2$ (aa 2106–2120) and used it to probe HEK 293T cells expressing $Ca_V1.2$. The C-terminal antibody (anti-CCAT) recognizes both the intact channel and a short cleavage product that corresponds to the C-terminal fragment. In contrast, an antibody recognizing an epitope in the II-III cytoplasmic loop of $Ca_V1.2$ (anti-II-III loop) detects full-length and C-terminally truncated channels only (Figure 1A).

To determine where CCAT is localized in cells in the brain, we purified nuclear, cytoplasmic, and membrane fractions of postnatal day 7 (P7) rat brain cortex and used western blotting to probe them with the anti-CCAT antibody (Figure 1B). Surprisingly, we found that the nuclear extracts contained high levels of CCAT, suggesting that the C terminus of $Ca_V1.2$ is localized in the nucleus of cells in the brain. In contrast, the N-terminal portion of the channel was localized in the membrane and cytoplasmic fractions, as expected for an ion channel. To provide further evidence that CCAT is indeed nuclear in neurons or glial cells, we examined its localization by immunostaining primary cortical cultures. The anti-CCAT antibody stained the cell body and dendrites of neurons weakly (Figure 1D), suggesting that the anti-CCAT antibody recognizes some intact $Ca_V1.2$ channels. Importantly, however, CCAT staining was observed in the nucleus of many neurons and was particularly pronounced in 10% of the cells (Figure 1C). In contrast, the II-III loop antibody stained the cell bodies and dendrites of neurons but was excluded from the nucleus, suggesting that the full-length channel is not nuclear (Figure 1E).

To investigate which types of neurons have nuclear CCAT, we costained neurons with anti-CCAT and with antibodies that stain precursor cells (nestin), glial cells (GFAP), excitatory neurons (VGLUT), or inhibitory neurons (GAD65) in the cortex. We found that cells that have strong nuclear CCAT also expressed glutamic acid decarboxylase (GAD65), suggesting that CCAT is strongly nuclear in inhibitory neurons that produce GABA (Figure 1F). To determine if CCAT is also in the nucleus of neurons in vivo, we used the anti-CCAT antibody to stain P30 rat brain sections. A subset of cells in the thalamus (data not shown), inferior colliculus (Figure 1G), inferior olivary nucleus (Figure 1H), and in the olfactory bulb (Figure 1I) displayed prominent nuclear CCAT staining. In the cortex and the hippocampus, CCAT was nuclear in a small number of neurons, consistent with its localization in a subset of GAD65-positive neurons in cortical cultures (data not shown). Taken together, these experiments indicate that CCAT is localized in the nucleus of inhibitory neurons, in culture and in restricted regions of the brain in vivo.

To provide further evidence that CCAT can translocate to the nucleus, we fused yellow fluorescent protein (YFP) to the C terminus of full-length $Ca_V1.2$ ($Ca_V1.2$-YFP). We observed cytoplasmic and nuclear fluorescence when $Ca_V1.2$-YFP was expressed in neurons (Figure 2A), cardiac myocytes (data not shown), or Neuro2A glioblastoma cells (Figure S2). In contrast, in neurons expressing $Ca_V1.2$ tagged at its N terminus with YFP, the channel was localized in the membrane and in the endoplasmic reticulum (Figure 2B). We did not observe nuclear fluorescence in HEK 293T cells expressing $Ca_V1.2$-YFP, consistent with previous reports that in HEK 293T cells the C terminus of $Ca_V1.2$ remains associated with the plasma membrane following cleavage (Gao et al., 2001; Gerhardstein et al., 2000; Hulme et al., 2005). However, a fusion of YFP and the last 503 amino acids of $Ca_V1.2$ was nuclear and formed distinct nuclear punctae in neurons, myocytes, and HEK 293T cells (c503, Figures 2C and 2E). Interestingly, this punctate pattern did not seem to be the result of overexpression, as it was also observed in some neurons by confocal imaging of endogenous CCAT staining (Figure 2D) and it was enhanced by incubation in low calcium media (Figure 3B). These experiments provide further evidence that CCAT is indeed nuclear and suggest that formation of punctae by endogenous CCAT is modulated by signaling events in the cell.

Nuclear CCAT does not contain a canonical nuclear localization sequence, suggesting that it enters the nucleus via an alternative pathway, perhaps as has been described for Stat1 protein, where nuclear import is mediated by direct interaction with nucleoporins (Marg et al., 2004). To identify the regions of CCAT that are necessary for its nuclear localization, we made truncations of the 503-YFP protein and introduced them in HEK 293T cells. Deletion of the carboxyl end of CCAT and of amino acids 1642–1814 of $Ca_V1.2$ (c330) had little effect on the protein's localization. In contrast, deletion of amino acids 1814–1864 (c280) decreased nuclear retention and abolished punctae formation (Figures 2E and 2F). Comparison of the $Ca_V1.2$ sequence from other vertebrates indicates that this nuclear retention domain is conserved evolutionarily (Figure S1A), suggesting that it plays an important role in the function of $Ca_V1.2$ and CCAT proteins.

Endogenous CCAT is predicted to be a 75 kDa protein; therefore, nuclear translocation of CCAT is likely to involve an active process rather than passive diffusion across nuclear pores. To estimate the rate of CCAT import into the nucleus, we used fluorescence recovery after photobleaching (FRAP) and time-lapse microscopy of Neuro2A cells expressing $Ca_V1.2$-YFP. After photobleaching of nuclear CCAT, nuclear fluorescence recovered over the course of 300 s with a single exponential time course ($t = 48 \pm 16$ s; $n = 11$), while cytoplasmic fluorescence declined over the same time period (Figures 2G–2H). In control cells expressing YFP alone, we observed an almost instantaneous recovery of nuclear fluorescence after photobleaching, concomitant with a decrease in cytoplasmic fluorescence, consistent with the observation that