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Excitation-transcription coupling in sympathetic neurons and the molecular mechanism of its initiation

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Abstract

In excitable cells, membrane depolarization and activation of voltage-gated Ca^{2+} (Ca_V) channels trigger numerous cellular responses, including muscle contraction, secretion, and gene expression. Yet, while the mechanisms underlying excitation-contraction and excitation-secretion coupling have been extensively characterized, how neuronal activity is coupled to gene expression has remained more elusive. In this article, we will discuss recent progress toward understanding the relationship between patterns of channel activity driven by membrane depolarization and activation of the nuclear transcription factor CREB. We show that signaling strength is steeply dependent on membrane depolarization and is more sensitive to the open probability of Ca_V channels than the Ca^{2+} entry itself. Furthermore, our data indicate that by decoding Ca_V channel activity, CaMKII (a Ca^{2+}/calmodulin-dependent protein kinase) links membrane excitation to activation of CREB in the nucleus. Together, these results revealed some interesting and unexpected similarities between excitation-transcription coupling and other forms of excitation-response coupling.

Keywords

CaM/CaMKII; Excitation-transcription coupling; Phosphorylation of cAMP response element-binding protein (pCREB); Calcium channels

1. Introduction

Excitable cells, such as neurons, convert external stimuli into signals that produce a variety of biological responses. For example, calcium flux through voltage-gated Ca^{2+} channels triggers communication to the nucleus to regulate gene expression. This process, known as excitation-transcription (E-T) coupling, is less well understood than other relationships between channel activation and biological output. Indeed, excitation-contraction (E-C) coupling (Armstrong et al., 1972; Chapman and Tunstall, 1981; Hodgkin and Horowicz, 1960; Schneider, 1994) and excitation-secretion (E-S) coupling (Augustine, 2001; Augustine et al., 1985; Katz and Miledi, 1967; Llinas et al., 1981; Schneggenburger and Neher, 2005)

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have been intensely investigated for more than half a century. E-T coupling involves many modes of signal propagation (voltage, Ca^{2+}, proteins) over widely varying spatial and time scales. The most scrutinized example of E-T coupling is signaling to the transcription factor cAMP response element-binding protein (CREB) *via* phosphorylation at Ser133, which is critical for CRE-mediated gene expression and many adaptive changes in neurons (Carlezon et al., 2005; Lonze and Ginty, 2002). Compared to the other voltage-gated Ca^{2+} channels, Ca_V1 (also called L-type) channels enjoy a big advantage in such signaling over Ca_V2 channels (Deisseroth et al., 2003; Dolmetsch, 2003; Murphy et al., 1991; West et al., 2002), in part because of their private access to local Ca^{2+}-dependent signaling machinery (Deisseroth et al., 1996; Dolmetsch et al., 2001; Weick et al., 2003; Zhang et al., 2005). However, the molecular mechanisms that allow Ca_V1 channels to decode external cues and send information to the nucleus remain incompletely understood.

In this article, we summarize progress in understanding E-T coupling by addressing the following important questions: (1) What is the stimulus-response (input-output) relationship? (2) How steeply does E-T coupling depend on membrane depolarization, channel gating, and Ca^{2+} influx? Is it comparable to EC and E-S coupling? (3) How do Ca_V1 channels decode information from external stimuli? To address these fundamental issues, we have combined molecular and cellular approaches and electrophysiology with a multidisciplinary systems analysis of cultured superior cervical ganglion (SCG) neurons. Our results indicate that E-T coupling depends on Ca^{2+} channel activation in a steeply cooperative manner. This steepness arises from changes in Ca_V1 channel open probability (P_o), but not simply total Ca^{2+} influx. Furthermore, our data suggest that by deciphering Ca^{2+} channel activity, Ca^{2+}/calmodulin (CaM)-dependent protein kinase II (CaMKII) provides a molecular link between membrane depolarization and activation of CREB.

2. Importance of a time gap in studies of coupling between depolarization and CREB phosphorylation

Previous studies of excitation-transcription coupling have mostly used long exposures of K^+-rich solution, typically producing depolarizations lasting 30 min or more, to allow time for production of transcripts (Greenberg et al., 1986; Sheng et al., 1990). Our focus was on the initial events that trigger CREB phosphorylation (pCREB) and less on the later steps in gene activation. We found that stimulation with 40 mM K^+ for only a few seconds is sufficient to generate a clearly detectable pCREB signal in individual neurons, if the depolarization is followed by an appropriate (and surprisingly brief) delay (Wheeler et al., 2008). Such a pulse-delay protocol has two obvious advantages. First, it provides a stimulus duration comparable to that achieved by a series of transmitter-induced depolarizations or a burst of action potential firing (neurons seldom undergo sustained depolarizations lasting tens of minutes under physiological circumstances). Second, the protocol allows the exploration of the signaling under biophysical conditions, in which the signaling is most input-sensitive, an advantage for gaining mechanistic insights. This is comparable to studying ion channels operating at the foot of their activation curves (Almers, 1978). Figure 1 provides an illustration of the depolarization-induced increase of pCREB and the way that we optimized the delay. pCREB immuno-intensity in the nucleus was strongly increased by 40 mM K^+ stimulation for 180 s (Fig. 1A). With briefer stimulation, the pCREB response was very small if the cells were fixed immediately after the stimulation (Fig. 1B). However, if a delay was introduced between stimulation and fixation, the same stimulus could trigger a large pCREB response. By systematically varying the delay, we found that the largest pCREB response occurred with a delay of 45 s (Fig. 1B). Presumably, longer delays allow the depolarization-initiated signaling to gradually decay away while briefer delays leave less time for the signal to arrive at the nucleus (Deisseroth et al., 1998; Mermelstein et al., 2001).

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By keeping a 45 s gap (Fig. 1C), we were able to discern measurable pCREB responses as we varied the duration of stimulation over a range of seconds to tens of seconds.

3. Steep voltage-dependence of E-T coupling

Activation of muscle contraction and neurosecretion were analyzed by Alan Hodgkin, Bernard Katz and colleagues in pioneering studies dating back to the 1960s (Hodgkin and Horowicz, 1960; Katz and Miledi, 1967). A common feature of both E-C and E-S coupling are the steep relationships between changes in membrane voltage and biological output. This inspired us to ask whether there is also a similar relationship between excitation and transcription by utilizing pCREB immunocytochemistry as the readout of responses to K^+ depolarization in individual rat sympathetic neurons. Consistent with previous studies (O'Lague et al., 1974; Wheeler et al., 2006), the resting potential of SCG neurons in 5 mM K^+ was approximately −60 mV (Fig. 2A). Exposure to 20, 30, and 40 mM K^+ depolarized the cells to −37, −26, and −19 mV, respectively, following the Nernst relationship (Fig. 2A). Importantly, we found that although relatively weaker stimuli (e.g. 20-30 mM K^+) drove much shallower signaling to pCREB, the levels eventually approached those attained with 40 mM K^+ (Fig. 2B). To estimate the strength of signaling, we determined the per-unit-time effect of a given stimulus by measuring the initial slope of the time-dependent increase in pCREB (Fig. 2C). A very dramatic change in output was found with small gradations in input, demonstrating the inherent sensitivity of the signaling system. Furthermore, plotting pCREB signal strength against membrane voltage revealed a steep relationship of 5.6 mV/*e*-fold change (Fig. 2D), which is comparable to that found in E-C or E-S coupling (Fig. 2E, F).

4. P_o, not I_{Ca}, is the critical factor for E-T coupling strength

E-C and E-S coupling have been shown to share the generic feature of a steeply cooperative relationship between Ca^{2+} channel activation and biological response, but vary tremendously in their dependence on Ca^{2+} flux (Schneggenburger and Neher, 2005; Schneider, 1994). Indeed, whereas skeletal E-C coupling operates independently of Ca^{2+} influx through the plasma membrane (Armstrong et al., 1972; Beam and Franzini-Armstrong, 1997; Franzini-Armstrong and Protasi, 1997; Rios and Brum, 1987; Schneider and Chandler, 1973), E-S coupling is extremely sensitive to the magnitude of Ca^{2+} entry (Augustine et al., 1985; Bollmann et al., 2000; Dodge and Rahamimoff, 1967; Llinas et al., 1981; Schneggenburger and Neher, 2000; Sudhof, 2004). Having found that E-T coupling also relies on a steep relationship between Ca^{2+} channel activation and biological response, we asked what role Ca^{2+} flux played in this process.

To address this question, using voltage-clamp recordings we measured Ca^{2+} flux at voltages corresponding to given K^+ challenges and found that it displayed an exponential dependence on depolarization over the range we examined (from -37 to -19 mV) (Fig. 3A). Accordingly, we plotted pCREB signal strength against total Ca^{2+} flux for stimulations with 20, 30, and 40 mM K^+ (Fig. 3B). On a log-log scale, the data were fit by a straight line with a slope of 2.37, indicating that CREB signal strength was steeply dependent on total Ca^{2+} flux, which is the sum of the integrated Ca^{2+} flux through each individual channel, represented by the product of channel open probability (P_o) and unitary Ca^{2+} flux (I_{Ca}). Thus, to clarify the relationship between CREB signal strength and channel activity, it was important to determine the relative contribution of P_o and I_{Ca} to the pCREB signal. Voltage changes from -37 to -19 mV have little effect on Ca^{2+} driving force, and thus I_{Ca}; however, P_o changes steeply at this voltage range, suggesting that changes in P_o may underlie the steeply increasing signal strength. However, whether I_{Ca} also contributes to pCREB signal strength was still unknown. To address this question, we used Cd^{2+}, which has been shown to change

the permeability of the channel pore to Ca^{2+} on a microsecond time scale, thereby decreasing the integrated Ca^{2+} flux through the open channel (Chow, 1991; Lansman et al., 1986), to effect changes in I_{Ca} while keeping P_o fixed. As Figure 3D shows, even though Cd^{2+} potently blocked Ca^{2+} flux at -19 mV (equivalent to 40 mM K^+ stimulation) (Fig. 3C), it only had a mild effect on CREB signal strength: the regression line of I_{Ca} is much shallower than that for the gradations of P_o (slopes of 0.74 versus 2.37; Fig. 3D). This suggests that I_{Ca} is not the critical factor underlying the steeply increasing signal strength. Thus, for E-T coupling, the relationship between depolarization and signal strength is not a simple function of total Ca^{2+} flux, but is rather closely tied to voltage-dependent changes in P_o (Fig. 3D, E).

5. CaMKII is essential for E-T coupling

We previously found that depolarizing SCG neurons results in Ca_V1 channel-dependent phosphorylation of CREB and CRE-mediated gene expression (Wheeler et al., 2006; Wheeler and Cooper, 2001). Because the P_o of Ca_V1 channels is highly voltage dependent (Reuter et al., 1982), if E-T coupling is primarily determined by channel P_o then the next question is which molecule(s) are used to decode the pattern of Ca^{2+} transients near the Ca_V1 channel. An important Ca^{2+} sensor, CaM activates CaMKII in an activity-dependent manner; thus, CaMKII is extremely sensitive to Ca^{2+} spike frequency and selectively responds to high-frequency brief stimuli, such as Ca^{2+} fluxes at the mouth of ion channels (Kubota and Bower, 2001). It has been reported that activated CaMKII is important for signaling to CREB (Takeda et al., 2007), suggesting that CaMKII could be a logical candidate because it is tethered to Ca_V1 channels (Hudmon et al., 2005) and to Ca_V1 interacting proteins such as densin (Jenkins et al., 2010). Furthermore, autophosphorylation and persistent activation of CaMKII increase steeply with the frequency of Ca^{2+} pulses (De Koninck and Schulman, 1998).

If CaMKII is important for E-T coupling, direct evidence could come from its role in pCREB signaling. To test this, we examined pCREB formation after knocking down the expression of α and βCaMKII, the main CaMKII isoforms in neurons (Fig. 4A). Interestingly but not unexpectedly, signaling to the nucleus was reduced by >65% after CaMKII was knocked down (Fig. 4A, B), which indicates CaMKII is important for E-T coupling. However, whether CaMKII could decode the external information in E-T coupling was still unknown. To approach this issue, two critical questions were addressed. First, is CaMKII activated locally, near the source of Ca^{2+} entry (that is, near the Ca_V1 channel itself)? And second, is CaMKII also highly P_o-sensitive but only weakly I_{Ca}-sensitive? As Figure 4C shows, brief stimulation with 40 mM K^+ recruited autophosphorylation of CaMKII (pCaMKII) to form puncta near the cell surface, which is consistent with local CaMKII activation. Furthermore, a Ca_V1 channel inhibitor, nimodipine prevented the formation of these pCaMKII puncta upon stimulation, indicating that Ca_V1 channel activity is also required for CaMKII activation (Fig. 4D). By using Cd^{2+} to decrease I_{Ca}, we found that grading I_{Ca} had a much milder effect on CaMKII activation than changing P_o (Fig. 4E), similar to its effect on pCREB signaling (compare Figs. 4E and 3D). Taken together, these data suggest that in E-T coupling, CaM/CaMKII serves as a local Ca^{2+} sensor to help Ca_V1 channels decode the external inputs, a critical early step for signaling to the nucleus.

6. Conclusion

Activity-dependent changes in neuronal morphology, excitability, and synaptic strength are all dependent on gene expression and protein synthesis. Accordingly, a thorough understanding of the mechanisms by which neurons link membrane depolarization to nuclear signaling is of critical importance. It is well known that Ca_V1 channels can couple

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membrane depolarization to nuclear transcription (Greenberg et al., 1986; Morgan and Curran, 1986; Murphy et al., 1991), acting on local signaling machinery (Deisseroth et al., 1996; Dolmetsch et al., 2001; Oliveria et al., 2007; Weick et al., 2003). However, the downstream mechanisms following Ca^{2+} influx have remained unclear. In this article, we used sympathetic neurons as model cells to characterize the basic properties of E-T coupling and make comparisons with E-C and E-S coupling. We found that signal strength in E-T coupling is steeply dependent on Ca^{2+} channel activity, similar to E-C and E-S coupling (Fig. 2D-F). Importantly, E-T coupling strength is not simply a reflection of the integrated strength of Ca^{2+} influx. Instead, our experiments indicate that it is highly sensitive to P_o, but only weakly sensitive to I_{Ca}, even though P_o and I_{Ca} are both multiplicative factors in determining Ca^{2+} flux overall (Fig. 3D).

The P_o of Ca_V1 channels increases in a steeply voltage-dependent manner (Fig. 5A). On the other hand, local saturation of Ca^{2+} sensors in the immediate vicinity of an open channel would reduce the importance of changes in I_{Ca}, effectively transforming analog Ca^{2+} signals into a digital format. To support this hypothesis, we have determined that CaM/CaMKII serves as the Ca^{2+} sensor for decoding this digital frequency of Ca^{2+} transients near the channel. Activated CaMKII is in the immediate vicinity of Ca_V1 channels (Grueter et al., 2006; Hudmon et al., 2005; Jenkins et al., 2010) and is steeply dependent on the frequency of Ca^{2+}/CaM pulses (Fig. 5B). Three lines of evidence support its participation in signaling to the nucleus. First, decreasing CaMKII levels reduced the strength of CREB signaling (Fig. 4B). Second, pCaMKII puncta formed at the cell surface in a Ca_V1 channel activity-dependent manner (Fig. 4C, D). Third, pCaMKII formation was much more sensitive to changing P_o than I_{Ca} (Fig. 4E), thus accounting for the disparate effects of P_o and I_{Ca} in signaling to CREB. Taken together, our findings provide evidence that local CaMKII activation is critical in decoding Ca^{2+} transients generated by Ca_V1 channel openings, which is an initial step for E-T coupling in the sympathetic neurons.

Acknowledgments

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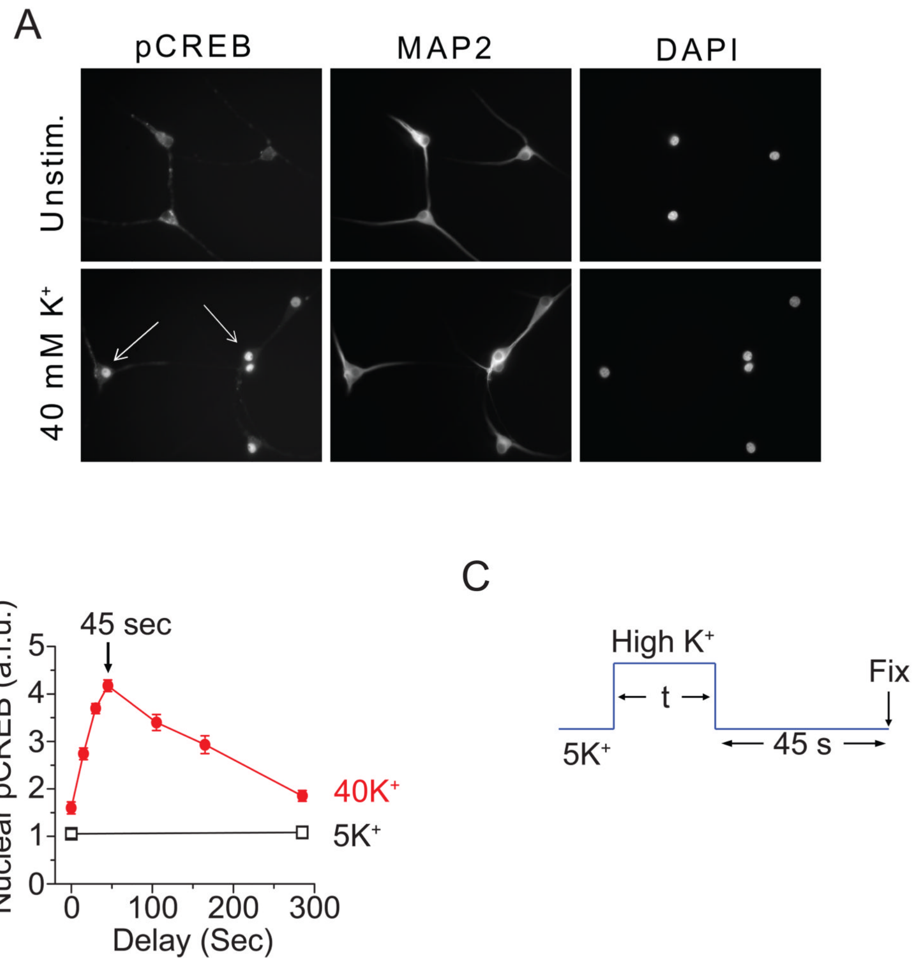

Fig. 1. Quantification of pCREB signaling with a time delay

(A) SCG neurons (cultured 5 days) were either mock-stimulated in 5 mM K^+ Tyrode solution (containing 2 mM Ca^{2+}) or stimulated with solutions containing 40 mM K^+ for 180 s. After stimulation, cells were immediately fixed and stained for pCREB and MAP2; nuclei were counterstained with DAPI. Arrows indicate strongly activated neurons. (B) Neurons were stimulated with 5 or 40 mM K^+ for 10 s followed by a variable time delay before fixation. Peak pCREB levels occurred 45 s after stimulation and decayed thereafter, becoming nearly indistinguishable from baseline within 5 min. (C) To ensure that the pCREB level measured after a given stimulation was a maximal response, we stimulated the cells for different time with a fixed 45 s delay in 5 mM K^+ Tyrode solution. (Modified from Wheeler et al., 2008 or Wheeler et al. unpublished data.)

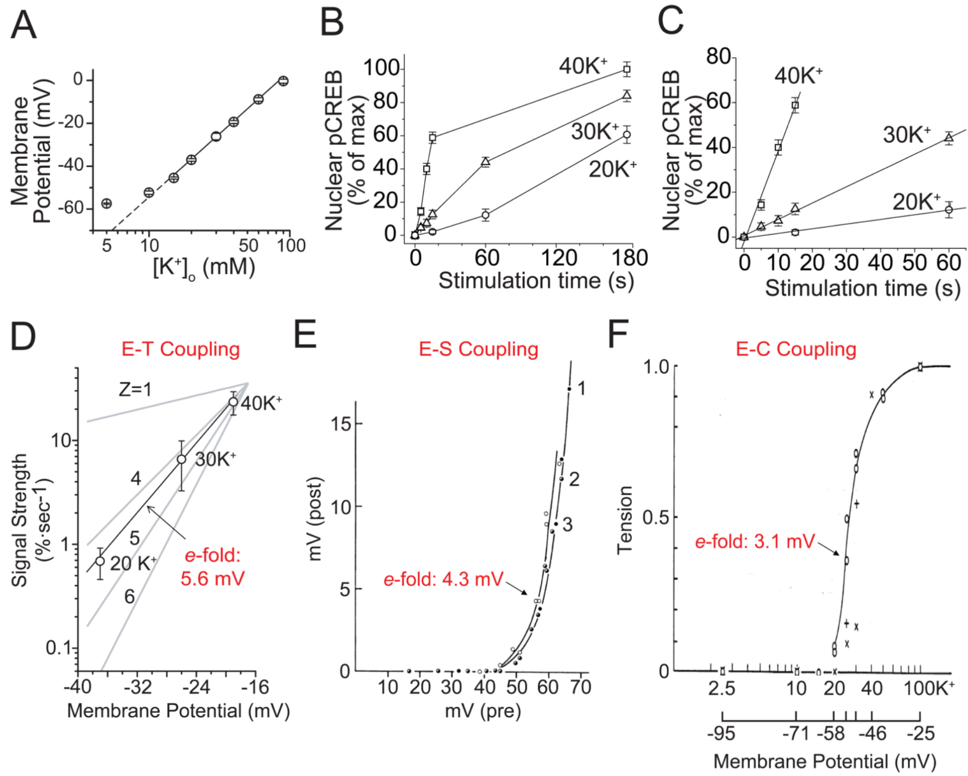

Fig. 2. Strength of signaling to CREB in SCG neurons

(A) Membrane potential plotted against $[K^+]_o$. The slope of the linear fit is 58.1 mV per 10-fold change in $[K^+]_o$, consistent with the Nernst equation. (B) Mean pCREB levels plotted against stimulation time (with a 45 s delay as determined in Figure 1); $[K^+]_o$ as indicated. (C) Linear fits of initial data points, using the slope as an index of CREB signal strength, pCREB. (D) CREB signal strength plotted against membrane voltage. 100% is the full change in pCREB from baseline to maximal levels. A linear fit shows that signal strength is steeply voltage dependent, as if governed by a gating particle with a valence (z) between 4 and 5 (corresponding to 5.6 mV/*e*-fold change in signal strength). (E) E-S coupling is also sharply dependent on voltage, with an e-fold change per 4.3 mV. Curves 1 (full circles), curves 2 (half-filled circles) and 3 (open circles) were obtained with pre-spikes, local pulses of 1 msec and 2 msec duration, respectively. (Katz et al., 1967). (F) The e-fold change for E-C coupling is per 3.1 mV (Hodgkin et al., 1960). (*A-D* Modified from Wheeler et al., 2008; *E* modified from Katz et al., 1967; *F* modified from Hodgkin et al., 1960.)

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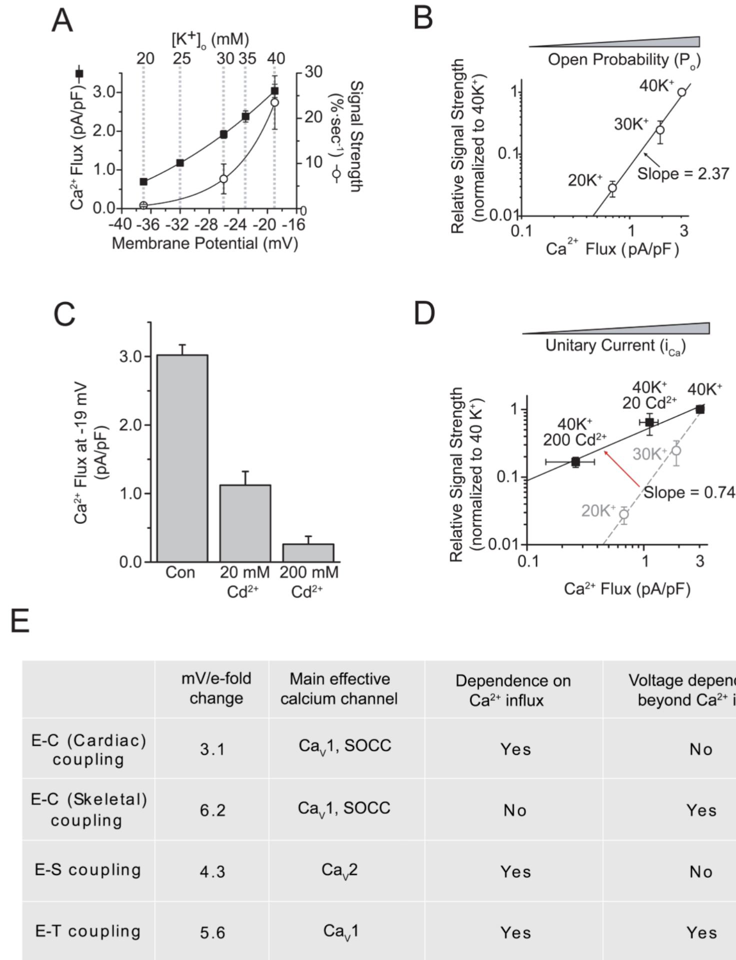

	mV/e-fold change	Main effective calcium channel	Dependence on Ca^{2+} influx	Voltage dependence beyond Ca^{2+} influx
E-C (Cardiac) coupling	3.1	Ca_V1, SOCC	Yes	No
E-C (Skeletal) coupling	6.2	Ca_V1, SOCC	No	Yes
E-S coupling	4.3	Ca_V2	Yes	No
E-T coupling	5.6	Ca_V1	Yes	Yes

Fig. 3. CREB signal strength is steeply dependent on channel Po but is largely independent of unitary Ca^{2+}flux

(A) Whole-cell Ca^{2+} flux (measured with 2 mM Ca^{2+} and normalized to cell capacitance) and CREB signal strength plotted versus membrane potential. Solid lines are single exponential fits. Dashed lines indicate the corresponding $[K^+]_o$ yielding the given membrane potentials, derived from Figure 2A. (B) Log-log plot of signal strength versus Ca^{2+} flux for the $[K^+]_o$ indicated.]The solid line is a linear fit of the data. (Almers) Whole-cell Ca^{2+} flux, in the presence of 0, 20, or 200 μM Cd^{2+} (Almers)CREB signal strength for 40 K^+ with 0, 20, or 200 μM Cd^{2+}. The solid line is a linear fit of the Cd^{2+} data. For comparison, the dashed line and gray data points are reproduced from B. (E) Some salient features of the three major forms of excitation-response coupling. *SOCC*, *store-operated calcium channel*. (Modified from Wheeler et al., 2008.)

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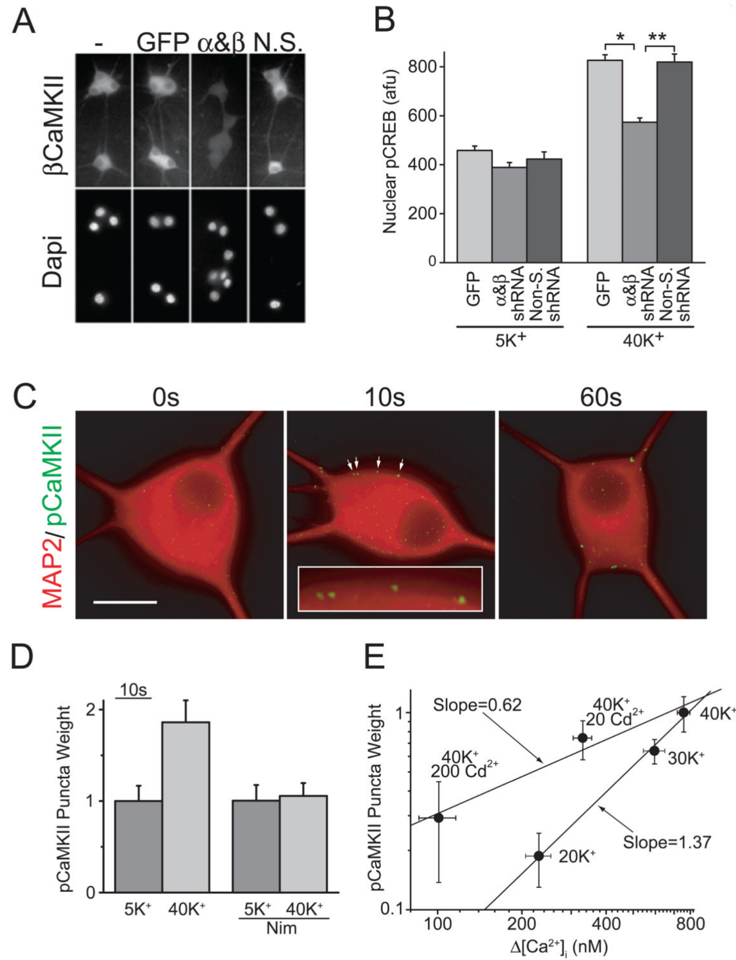

Fig. 4. CaMKII is critical for CREB signaling

(A) SCG neurons infected with lentiviruses expressing GFP alone, shRNAs against α and βCaMKII, or nonsilencing control shRNAs, and stained with an antibody against βCaMKII; nuclei were counterstained with DAPI. (B) pCREB levels from neurons stimulated for 2.5 s with 5 mM K^+ or 40 mM K^+ and transferred to a 5 mM K^+ solution for 45 s before fixation. *$p<5\times10^{-17}$; **$p< 5\times10^{-10}$. (C) SCG neurons stimulated with 40 mM K^+ for 0, 10, or 60 s and immediately fixed and stained for MAP2 (red) and pCaMKII (green). Arrows point to pCaMKII puncta (magnified in the inset). Background pCaMKII staining was subtracted to highlight punctuate staining. Scale bar, 20 μm. (D) Nimodipine blocks pCaMKII puncta formation. (E) Plot of pCaMKII puncta weight versus the rise in bulk Ca^{2+} measured using Fura-2 ratiometric Ca^{2+} imaging for the stimulation conditions indicated. (Modified from Wheeler et al., 2008.)

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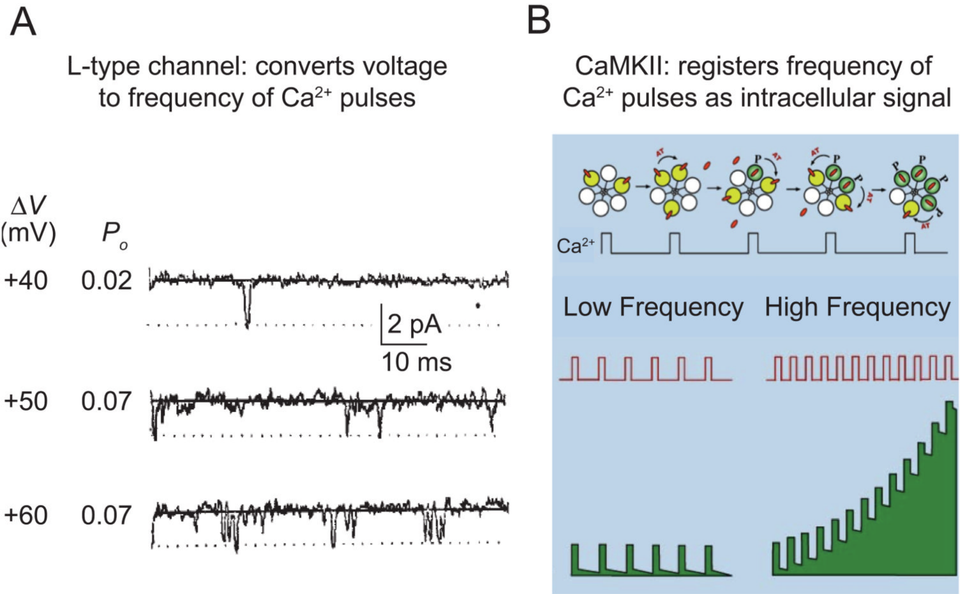

Fig. 5. CaM/CaMKII decodes the digital frequency code generated by Ca_V1 channels

(A) The frequency of Ca_V1 channel opening (P_o) is steeply voltage-dependent (Reuter et al., 1982). (B) The activity of CaMKII increases in a steeply frequency-dependent way (top) (De Koninck and Schulman, 1998). Quantitative modeling suggests that the frequency-dependence holds for brief pulses of Ca^{2+} of the kind produced by the activity of Ca_V1 channels (Dupont et al., 2003). Thus, the combination of Ca_V1 and CaMKII supports a highly sensitive transduction of cell membrane potential to intracellular biochemical signaling (bottom). (*A* modified from Reuter et al., 1982; *B* was kindly provided by A. Hudmon.)