Ca²⁺ channel nanodomains boost local Ca²⁺ amplitude

Michael R. Tadross^{a,b,c,1}, Richard W. Tsien^{b,d,1,2}, and David T. Yue^{c,e,2}

^aJanelia Farm Research Campus, Howard Hughes Medical Institute, Ashburn, VA 20147; ^bDepartment of Molecular and Cellular Physiology, Beckman Center, Stanford University School of Medicine, Stanford, CA 94305; ^cCalcium Signals Laboratory, Department of Biomedical Engineering, The Johns Hopkins University School of Medicine, Baltimore, MD 21205; ^dLangone Medical Center Neuroscience Institute, New York University, New York, NY 10016; and ^eCalcium Signals Laboratory, Department of Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, MD 21205

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Local Ca²⁺ signals through voltage-gated Ca²⁺ channels (Ca_vs) drive synaptic transmission, neural plasticity, and cardiac contraction. Despite the importance of these events, the fundamental relationship between flux through a single Ca_v channel and the Ca²⁺ signaling concentration within nanometers of its pore has resisted empirical determination, owing to limitations in the spatial resolution and specificity of fluorescence-based Ca²⁺ measurements. Here, we exploited Ca²⁺-dependent inactivation of Ca_v channels as a nanometer-range Ca²⁺ indicator specific to active channels. We observed an unexpected and dramatic boost in nanodomain Ca²⁺ amplitude, ten-fold higher than predicted on theoretical grounds. Our results uncover a striking feature of Ca_v nanodomains, as diffusion-restricted environments that amplify small Ca²⁺ fluxes into enormous local Ca²⁺ concentrations. This Ca²⁺ tuning by the physical composition of the nanodomain may represent an energy-efficient means of local amplification that maximizes information signaling capacity, while minimizing global Ca²⁺ load.

signaling | uncaging | biosensor | electrodiffusion

Local Ca²⁺ signals increase the information capacity of signaling within a cell, by enabling short-range signals to operate independently of Ca²⁺ events elsewhere throughout the cell (1, 2). These local Ca²⁺ signals are the conduit that drives diverse activity-dependent events, such as synaptic transmission (3), neural plasticity (4–6), and cardiac excitation–contraction coupling (7). However, with regard to the Ca²⁺ amplitude within nanometers of individual Ca²⁺ channels, our knowledge is based predominantly on diffusion theory, which predicts ~100 μM Ca²⁺ signals per picoampere flux at a distance of ~10 nm from the pore (8–11). Although often quoted, this theory has been difficult to verify experimentally because diffusible Ca²⁺ indicators report space-averaged [Ca²⁺], and thus lack the spatial resolution needed for selective nanodomain reporting. Two recent efforts to overcome this limitation used voltage-gated Ca²⁺ channel (Ca_v)-tethered fluorescent Ca²⁺ indicators to isolate nanodomain signals (12, 13), but were met with unexpected difficulties: Ca_v-tethered indicators were unresponsive in the presence of high concentrations of intracellular 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), a Ca²⁺ buffer that preserves Ca²⁺ elevations within tens of nanometers of active channels but eliminates it elsewhere (8–10). A lack of response under these conditions would arise if the majority of tethered channels were silent, raising the concern that fluorescence changes observed under milder Ca²⁺ buffering (12, 13) may represent the response of indicators tethered to silent channels, which nonetheless eavesdrop on Ca²⁺ from active channels nearby. To overcome this fundamental limitation, we used a reverse strategy, whereby Ca²⁺/calmodulin-dependent inactivation (CDI) of channels themselves serves as a nanometer-range indicator of [Ca²⁺]; a sensitive ionic readout that is unaffected by the presence of silent channels, and exquisitely specific to [Ca²⁺] in the nanodomain of active channels.

Our strategy was to calibrate CDI with spatially uniform Ca²⁺ uncaging, and then use CDI to measure Ca_v-generated Ca²⁺ signals. We chose Ca_v1.3 because it displays prominent CDI, driven by Ca²⁺ binding to calmodulin (CaM), an indwelling subunit of

the channel (14–16). To calibrate CDI, we generated Ca²⁺ elevations via intracellular Ca²⁺ uncaging, while monitoring CDI via the flux of Li⁺ ions, which do not bind to CaM or Ca²⁺ indicators (Fig. 1*A*). We engineered the selectivity filter of the channel to favor the flux of Li⁺ permeation in the face of Ca²⁺ uncaging (refs. 17, 18; *SI Appendix, Fig. S1*), and restricted Ca²⁺ binding to the C lobe of CaM₁₂ to isolate the simplest form of CDI (16).

Because photolysis is spatially uniform, the free Ca²⁺ concentration in the nanodomain will equal Ca²⁺ elsewhere within the cell, where it can be accurately determined with ratiometric indicators (19–21) (Fig. 1*B, Middle row; Materials and Methods*; and *SI Appendix, Fig. S1*). Before uncaging, step depolarization (Fig. 1*B, Top*) produced noninactivating Li⁺ currents (Fig. 1*B, Bottom, black trace*), consistent with an absence of voltage-dependent inactivation (22). Uncaging to low [Ca²⁺] evoked a small amount of inactivation (Fig. 1*B, Left, red trace*), and steps to higher [Ca²⁺] resulted in stronger CDI (Fig. 1*B, Right*). In parallel experiments, CDI was virtually eliminated with a Ca²⁺-insensitive CaM₁₂₃₄ (23, 24) (Fig. 1*C and D*). This confirmed that uncaged Ca²⁺ had little direct effect on Li⁺ permeation, and enabled determination of CDI mediated entirely by Ca²⁺ binding to the C lobe of CaM (*SI Appendix, Fig. S1*). Pooled data from 51 cells followed a tight binding curve with half-maximal effective concentration, $EC_{50} = 2.2 \pm 0.1 \mu\text{M}$ and Hill coefficient $n = 1.5 \pm 0.1$ (Fig. 1*E*). This procedure was tantamount to the familiar live-cell calibration of a fluorescent Ca²⁺ indicator, with the distinction that because CDI is an ionic measure, it will be precisely specific to active (rather than silent) Ca_v channels.

We turned next to CDI mediated by local Ca²⁺ elevations generated by flux through Ca_v channels (Fig. 2*A*). A graded degree of CDI was observed with variations of external Ca²⁺ concentration, using Ca²⁺ as the only external charge carrier. CDI averaged ~50% at 0.05 mM [Ca²⁺]_o (Fig. 2*B*). As before, we

Significance

Local Ca²⁺ signals drive important events like synaptic transmission, neural plasticity, and cardiac contraction, yet the fundamental relationship between flux through a single channel and the Ca²⁺ amplitude within nanometers of the channel pore has eluded direct experimental measure. Here we reverse-engineer the problem by using Ca²⁺-dependent inactivation of channels as a nanometer-range biosensor of Ca²⁺ amplitude. We discovered an unexpected and dramatic boost in nanodomain Ca²⁺ amplitude, ten-fold higher than predicted on theoretical grounds. This boost in local Ca²⁺ signaling may act to maximize the biochemical information capacity of electrically-active cells.

Author contributions: M.R.T. conceived, designed, and performed all experiments, analyses, and simulations. M.R.T., R.W.T., and D.T.Y. wrote the paper.

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¹To whom correspondence may be addressed. E-mail: tadrossm@janelia.hhmi.org or Richard.Tsien@nyumc.org.

²R.W.T. and D.T.Y. contributed equally to this work.

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Fig. 2. Measuring Ca_v -generated local Ca^{2+} amplitude. (A) Schematic use of CDI to measure nanodomain Ca^{2+} amplitude. (B) Local Ca^{2+} -mediated CDI data. Colored traces correspond to external solutions with Ca^{2+} as the only charge carrier. These contained 40 (red), 0.6 (orange), 0.15 (green), and 0.05 mM (blue) external $[\text{Ca}^{2+}]$ (Materials and Methods). Black trace corresponds to 40 mM Ba^{2+} data, a reference for voltage-dependent inactivation. Intracellular solutions contained 10 mM BAPTA. Traces show mean $\pm$ SEM of 5–22 cells for each condition. CDI is the fraction of current lost after 800 ms (as in Fig. 1B). To counterbalance surface charge screening, all step depolarizations were chosen 30 mV positive to $V_{1/2}$, the voltage of half-activation (SI Appendix, Fig. S2). (C) Single-channel data. Voltage ramps (Top) evoked $\text{Ca}_v1.3$ single-channel Ba^{2+} currents (gray traces). Patches containing a single channel were identified by currents entirely bounded by the closed- (zero line) and open-channel (dashed line) levels (SI Appendix, Fig. S2). Hundreds of sweeps from five patches were averaged to yield $\langle i_{\text{Ba}} \rangle$, the average single-channel flux in 40 mM Ba^{2+} (solid black curve overlaid with bottom sweep). At the relevant step potential ($V_{1/2} + 30$ mV), $\langle i_{\text{Ba}} \rangle = 0.27$ pA (black circle). (D) Whole-cell conversions. An identical ramp protocol (compare with C) was used in the whole-cell configuration. Black trace (40 mM Ba^{2+} data) provided a reference whole-cell current (i_{WC}) representing $N \cdot \langle i_{\text{Ba}} \rangle$, where N is the number of channels. Data from a given cell shared a common N , determined from the ratio of whole-cell to single-channel Ba^{2+} current. N typically ranged from 1,000 to 5,000. Scaling by this value (i_{WC}/N) converted whole-cell Ba^{2+} data to single-channel $\langle i_{\text{Ba}} \rangle$ amplitude (black symbol; 0.27 pA at $V_{1/2} + 30$ mV), and whole-cell Ca^{2+} data to single-channel $\langle i_{\text{Ca}} \rangle$ amplitude (colored symbols). CDI was eliminated via coexpression of Ca^{2+} -insensitive CaM_{1234} , and voltages were specified relative to $V_{1/2}$ (SI Appendix, Fig. S2). Traces show mean $\pm$ SEM of seven cells. (E) Average single-channel Ca^{2+} flux, $\langle i_{\text{Ca}} \rangle$, at $V_{1/2} + 30$ mV (D) depended on external Ca^{2+} according to a binding relation with $EC_{50} \sim 2$ mM and Hill coefficient ~ 1 . Error bars, when larger than symbol size, show SEM, where error estimates were propagated through each numerical step. Note log scales. (F) CDI (as in B) plotted as a function of $\langle i_{\text{Ca}} \rangle$ (as in E) for each condition. Error bars show SEM. Data followed a binding relation with $El_{50} = 2.15 \pm 0.43 \times 10^{-3}$ pA and Hill coefficient $n = 1.5 \pm 0.1$.

higher than expected, but also quite susceptible to buffering, inconsistent with the close-CaM hypothesis.

To reconcile these observations and their implications for Ca^{2+} signaling, we turned to classical Neher–Stern diffusion theory (8, 9), which predicts that in the nanodomain of an open channel, the $[\text{Ca}^{2+}]/\text{flux}$ ratio (G_0 , open-channel gain) depends on two factors, reflecting dispersion and buffering:

$$G_0 = \frac{[\text{Ca}^{2+}]}{i_{\text{Ca}}} = \underbrace{\frac{1}{4\pi \cdot F \cdot D_{\text{Ca}} \cdot r}}_{\text{dispersion}} \cdot \underbrace{\exp\left(\frac{-r}{\lambda}\right)}_{\text{buffering}} \quad \lambda = \sqrt{D_{\text{Ca}} / (k_{\text{B,on}} \cdot B_{\text{T}})}, \quad [1]$$

where r is distance from the channel pore, D_{Ca} is the diffusion coefficient of free Ca^{2+} ions, and F is Faraday's constant. In the absence of buffering, dispersion alone would produce a $1/r$ spatial gradient, reflecting the dilution of Ca^{2+} as it diffuses into hemispherical annuli of growing volume (Eq. 1 and Fig. 3B, dashed green curve). The buffering factor incorporates a length constant (λ , Eq. 1), reflecting the interplay between diffusion and buffering. Ca^{2+} buffers can intercept Ca^{2+} ions, but only with finite kinetics, specified by $k_{\text{B,on}}$ (buffer association rate), and B_{T} (total concentration of diffusible intracellular buffer). Thus, buffering has no efficacy at $r = 0$ where new ions enter the cytoplasm for the first time, but grows in influence as time from first entry and distance from the pore increase (Fig. 3B, dashed black and gray curves). Eq. 1 holds for virtually the entire

period of a channel opening because compact nanodomain dimensions ensure that Ca^{2+} influx, diffusion, and buffering all reach steady state within microseconds of channel opening (SI Appendix, Fig. S4). Similarly, $[\text{Ca}^{2+}]$ drops to baseline levels (~ 0 mM with BAPTA present) within microseconds once the channel shuts (10, 11, 16). Taking account of the duty cycle of channel opening and closing characterized by channel open probability (P_0), and the cooperativity of CDI reflected by Hill coefficient (n), the integrated gain (G , derived in SI Appendix, section III) is given by

$$G = \kappa \cdot G_0 \quad \kappa = P_0(1 - n)/n, \quad [2]$$

where κ , a correction factor for the intermittency of channel opening, would be unity if $P_0 = 1$, and greater than unity if $P_0 < 1$. In our case, $P_0 = 0.43$ was measured from single-channel data (SI Appendix, Fig. S2), $n = 1.5$ was obtained from the steepness of CDI-binding relations (Figs. 1E, 2F, and 3A), and κ was thus determined to be ~ 1.3 .

Reckoning with channel intermittency enabled direct comparison of theory to data. Thus, our experimental measurements of G (Fig. 3C, blue and red horizontal lines) are at odds with spatial profiles predicted by diffusion theory with conventional parameter values (Eqs. 1 and 2), which require CaM to be very close to the pore with 10 mM BAPTA but farther away with 60 mM BAPTA (Fig. 3C, blue and red arrows misaligned), a highly implausible scenario. The conflict would be resolved if the diffusion coefficient (D_{Ca}) were reduced 10-fold below that of free

Fig. 4. Potential modes of physiological tuning. (A) Model of localized diffusion restriction extending over radius r_{nano} from the pore, with normal diffusion at a distance. (B) Resulting G_O profiles. Green profiles correspond to homogeneous 1x and 0.1x profiles (replicated from Fig. 3B). Blue profiles correspond to conditions where the region of 0.1x diffusion is spatially isolated to $r_{\text{nano}} = 20$ or 35 nm (SI Appendix, Fig. S7). Local Ca^{2+} gain can be amplified by increasing the degree of diffusion restriction near the channel (vertical arrow). The spatial extent of amplification could be adjusted by modulating the radius of diffusion restriction (horizontal arrow).

synaptic transmission (19–21) as biosensors for presynaptic Ca^{2+} amplitude. Given these measures, canonical estimates of D_{Ca} have typically been required to infer distance from Ca_V to sensor. Here, we expand upon the basic biosensor approach to enable direct estimates of both D_{Ca} and r_{sensor} in the same preparation (Fig. 3F). In contrast to canonical parameters, our data indicate that Ca_V channels can amplify tiny Ca^{2+} fluxes into enormous Ca^{2+} concentrations, on the order of ~ 1 mM per pA (Fig. 3B, solid green curve).

Our findings may have implications in diverse physiological contexts. With regard to biochemical signaling cascades, tiny $\sim \text{fA}$ Ca^{2+} fluxes, generally believed not to partake in local signaling, could produce $\sim \mu\text{M}$ Ca^{2+} signals sufficient to signal locally to molecules like CaM or CaMKII (34). Nanodomain boosting may similarly play a role in the contraction of ventricular myocytes, where controversy exists regarding whether activation of ryanodine receptors can be triggered by the opening of a single Ca_V channel [high-fidelity scenario supported by early experimental work (35–38)], or if the simultaneous opening of ~ 10 channels is necessary [as suggested by recent models that take account of the small $\sim \text{fA}$ flux through a single Ca_V channel at the +50 mV ventricular plateau potential (39–41)]. Our findings may reconcile this low-flux high-fidelity paradox given that a single Ca_V channel would, by virtue of nanodomain Ca^{2+} boosting, have the potency of ~ 10 channels in the absence of this amplification. Similar implications may apply to presynaptic physiology, where the Ca^{2+} dependence of transmitter release has been experimentally determined (20, 21, 42, 43), and the sensitivity to exogenous buffers (EGTA and BAPTA) has been characterized in several preparations (44, 45). Whereas these findings are typically interpreted in the context of classical diffusion parameters, our findings may bear upon inferences of Ca_V -vesicle distance and stoichiometry.

With regard to mechanism, nanodomain Ca^{2+} boosting is most likely mediated by physical barriers to diffusion, termed tortuosity (28, 29), that slow the passage of free Ca^{2+} ions. Other potential

means of diffusion reduction (e.g., fixed buffers), which limit movement by binding/immobilization of Ca^{2+} ions could only lower the concentration of free Ca^{2+} in the nanodomain, and thus are unlikely to play a role in our findings (SI Appendix, Fig. S4). Of note, the 10-fold reduction in diffusion of unbound Ca^{2+} ions implicated here by local reporting far exceeds the twofold attenuation previously advanced for bulk cytosol (46–48). On this view, the domain within close range of the channel is a remarkably diffusion-restricted environment, where a Ca^{2+} ion would collide with an obstacle in nine out of ten Brownian movements. This energy-efficient means of local amplification seems ideally suited to address a universal challenge of Ca^{2+} homeostasis: the toxicity of global Ca^{2+} overload, and energetic cost of Ca^{2+} extrusion. By locally amplifying Ca^{2+} signals produced by diminutive fluxes, nanodomains likely facilitate the maintenance of numerous independent Ca^{2+} sources, maximizing information signaling capacity, and minimizing global Ca^{2+} load.

It remains to be determined exactly how far the region of amplification extends and what the precise nature of the diffusion barriers might be. One possibility is that Ca_V cytosolic domains may physically impede diffusion over a ~ 15 nm radius (SI Appendix, Fig. S7 and Fig. 4), as would be the case if Ca^{2+} ions passed through narrow Ca_V cytosolic crevices or portals (13, 49). Such a self-sufficient design would be robust to cellular context, and spatially isolated to the indwelling (50) and locally enriched pool (51) of CaM molecules mediating CDI and implicated in signaling to nuclear transcription factors (4, 5). Alternatively, the region of amplification could extend beyond the Ca_V itself, if augmented by diffusion-impeding assemblies of associated components. In this regard, it is interesting to note that Ca_V subtypes interact with a tremendous variety of proteins in different cellular contexts (52, 53). Indeed, the very same molecules that aggregate near Ca_V channels to convey downstream signals could also act to restrict diffusion, thereby increasing the gain between Ca^{2+} flux and biochemical response. It is similarly feasible that nanodomain boosting could be physiologically tuned by altering the density of associated components (Fig. 4B, vertical arrow, gain amplitude), or the radius over which associated components extend (Fig. 4B, horizontal arrow, gain radius). Such gain modulation would not only alter Ca^{2+} amplitude, but could also sharpen the Ca^{2+} profile, either by boosting Ca^{2+} in a restricted locale (Fig. 4) or by enhancing the efficacy of endogenous buffers like parvalbumin (54) (Fig. 3B). Thus, beyond amplification, diffusion-restricted nanodomains could play a role in curtailing crosstalk between Ca^{2+} sources, much like dendritic spines prevent Ca^{2+} spillover between adjacent postsynaptic sites (55).

Materials and Methods

Detailed methods can be found in SI Appendix. In brief, Rat α_{1D} (14) was engineered (E716A) to enhance permeation of Li^+ over Ca^{2+} (17, 18), and expressed in HEK293 cells as described (23). This mutant was used in all experiments. Whole-cell and single-channel recordings were as described (16). Ca^{2+} uncaging (Fig. 1), with a Cairn UV flashlamp, was performed 3–6 min after break-in; internal solutions contained Ca^{2+} -loaded DMNP-EDTA (1–4 mM). Uncaging transients were minimized with citrate (1–50 mM), zero Mg^{2+} , and zero ATP. Internal solutions were supplemented with phosphatidylinositol 4,5-bisphosphate and okadaic acid to stabilize Ca_V currents in the absence of Mg^{2+} /ATP. To enable ratiometric monitoring of $[\text{Ca}^{2+}]_i$, internal solutions contained a single-wavelength Ca^{2+} indicator (Fluo4FF) and red-shifted dye (Alexa568).

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