Elementary Mechanisms Producing Facilitation of Ca_{V2.1} (P/Q-type) Channels

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The regulation of Ca_{V2.1} (P/Q-type) channels by calmodulin (CaM) showcases the powerful Ca^{2+} decoding capabilities of CaM in complex with the family of Ca_{V1-2} Ca^{2+} channels. Throughout this family, CaM does not simply exert a binary on/off regulatory effect; rather, Ca^{2+} binding to either the C- or N-terminal lobe of CaM alone can selectively trigger a distinct form of channel modulation. Additionally, Ca^{2+} binding to the C-terminal lobe triggers regulation that appears preferentially responsive to local Ca^{2+} influx through the channel to which CaM is attached (local Ca^{2+} preference), whereas Ca^{2+} binding to the N-terminal lobe triggers modulation that favors activation via Ca^{2+} entry through channels at a distance (global Ca^{2+} preference). Ca_{V2.1} channels fully exemplify these features; Ca^{2+} binding to the C-terminal lobe induces Ca^{2+}-dependent facilitation of opening (CDF), whereas the N-terminal lobe yields Ca^{2+}-dependent inactivation of opening (CDI). In mitigation of these interesting indications, support for this local/global Ca^{2+} selectivity has been based upon indirect inferences from macroscopic recordings of numerous channels. Nagging uncertainty has also remained as to whether CDF represents a relief of basal inhibition of channel open probability (P_o) in the presence of external Ca^{2+}, or an actual enhancement of P_o over a normal baseline seen with Ba^{2+} as the charge carrier. To address these issues, we undertake the first extensive single-channel analysis of Ca_{V2.1} channels with Ca^{2+} as charge carrier. A key outcome is that CDF persists at this level, while CDI is entirely lacking. This result directly upholds the local/global Ca^{2+} preference of the lobes of CaM, because only a local (but not global) Ca^{2+} signal is here present. Furthermore, direct single-channel determinations of P_o and kinetic simulations demonstrate that CDF represents a genuine enhancement of open probability, without appreciable change of activation kinetics. This enhanced-opening mechanism suggests that the CDF evoked during action-potential trains would produce not only larger, but longer-lasting Ca^{2+} responses, an outcome with potential ramifications for short-term synaptic plasticity.

INTRODUCTION

The regulation of Ca_{V2.1} (P/Q-type) channels by calmodulin (CaM) holds special fascination for neurobiologists and ion channel biophysicists alike. As these channels represent the dominant trigger of neurotransmitter release in the central nervous system (Wheeler et al., 1994; Dunlap et al., 1995; Wheeler and Tsien, 1999), their CaM-mediated regulation is considered a potentially important mechanism for short-term synaptic plasticity (Borst and Sakmann, 1998; Cuttle et al., 1998; Xu and Wu, 2005), and thereby the neurocomputation of neural networks (Tsodyks and Markram, 1997; Tsodyks et al., 1998; Zucker and Regehr, 2002; Abbott and Regehr, 2004). From the channel biological viewpoint, this modulatory system prominently showcases the powerful Ca^{2+} decoding capabilities of CaM in complex with Ca_{V1-2} voltage-gated Ca^{2+} channels. There are three notable distinctives of this system. First, CaM in its Ca^{2+}-free form (apoCaM) is already preassociated with Ca_{V2.1} channels (DeMaria et al., 2001; Erickson et al., 2001); CaM is therefore poised to respond to Ca^{2+} elevations as a resident Ca^{2+} sensor. Second, subsequent Ca^{2+} binding to this resident CaM does not simply exert a binary on/off regulatory effect on Ca_{V2.1} channels. Rather Ca^{2+} binding to the C-terminal lobe of CaM selectively induces conformational changes that yield a facilitation of channel opening occurring over tens of milliseconds (Ca^{2+}-dependent facilitation [CDF]), whereas Ca^{2+} binding to the N-terminal lobe of CaM preferentially triggers alternate molecular changes that inactivate channel opening over the course of hundreds of milliseconds (Ca^{2+}-dependent inactivation [CDI]) (Lee et al., 1999; DeMaria et al., 2001; Lee et al., 2003; Chaudhuri et al., 2004). This CaM lobe–specific signaling represents an extreme form of the bipartitioning capabilities first observed in Paramecia (Kink et al., 1990). Third, though the two lobes of preassociated CaM are no doubt contained within the cytoplasmic nanodomain of channels (Augustine et al., 2003), they nonetheless seem to respond to different Ca^{2+} signal

Abbreviations used in this paper: APW, action-potential waveform; CaM, calmodulin; CDF, Ca^{2+}-dependent facilitation of opening; CDI, Ca^{2+}-dependent inactivation of opening; VDI, voltage-dependent inactivation process.
components embedded within the fluctuations of Ca$^{2+}$ concentration within this same nanodomain (Liang et al., 2003). Ca$^{2+}$ binding to the C-terminal lobe of CaM triggers regulatory processes (e.g., CDF in CaV2.1) that seem to respond preferentially to intense, spike-like Ca$^{2+}$ fluctuations driven by local Ca$^{2+}$ influx through the channel to which resident CaM is attached (local Ca$^{2+}$ preference) (Lee et al., 2000; DeMaria et al., 2001; Soong et al., 2002; Chaudhuri et al., 2004; Chaudhuri et al., 2005). By contrast, Ca$^{2+}$ binding to the N-terminal lobe activates modulatory processes (e.g., CDI in CaV2.1) that appear selective for smaller but long-lasting Ca$^{2+}$ components reflective of Ca$^{2+}$ entry through multiple other Ca$^{2+}$ channels at a distance from the resident CaM (global Ca$^{2+}$ preference). Though the precise form of modulation (CDF or CDI) produced by a particular lobe of CaM may vary in different types of Ca$^{2+}$ channels, the overall preference of the C-terminal lobe of preassociated CaM for local Ca$^{2+}$, and of the N-terminal lobe for global Ca$^{2+}$, appears to hold true across the family of Ca$_{v}$1.2 channels (Evans and Zamponi, 2006).

Ongoing biological research is beginning to reveal the impact of CaM/Ca$^{2+}$ channel regulation on cardiac (Alseikhan et al., 2002) and neuronal excitability (Chaudhuri et al., 2004; Chaudhuri et al., 2005; Yang et al., 2006). This study focuses instead upon critical gaps in our understanding of the mechanisms underlying CaM regulation of Ca$_{v}$2.1. A first key uncertainty concerns the suggested selectivity of CaM/channel regulation for spatially different sources of Ca$^{2+}$ influx. The data in support of this selectivity have thus far been indirect and based upon inferences drawn from macroscopic currents recordings from numerous channels. Whether this intriguing design principle actually exists could be tested directly at the single-channel level, where the local Ca$^{2+}$ signal is present, but the global Ca$^{2+}$ signal must be lacking by definition. A second critical unknown is whether Ca$_{v}$2.1 CDF represents a derepression of channel open probability ($P_{o}$), or an actual enhancement of $P_{o}$ over a normal baseline level seen without Ca$^{2+}$. Though either scenario would cohere with the macroscopic profile of CDF, direct determination of $P_{o}$ by single-channel recording would definitively adjudicate this ambiguity. Thus far, however, single-channel data for Ca$_{v}$2.1 channels have been limited primarily to those obtained with Ba$^{2+}$ as the charge carrier (Dove et al., 1998; Hans et al., 1999; Colecraft et al., 2001; Tottene et al., 2002; Fellin et al., 2004; Luvisetto et al., 2004; Tottene et al., 2005), where experimental resolution of signals is aided by the amplification of unitary current size (i) compared to that seen with the physiological charge carrier Ca$^{2+}$ (Tottene et al., 2002). Rapid millisecond openings of unitary Ca$_{v}$2.1 Ca$^{2+}$ currents are estimated to produce currents of only 300–400 femtoamps in amplitude (even with ~100 nM Ca$^{2+}$ as charge carrier); this challenging regime is at the cusp of practical experimental resolution by patch clamp.

Nonetheless, to address these and other open questions, we here undertake the first extensive single-channel analysis of Ca$_{v}$2.1 channels with Ca$^{2+}$ as the charge carrier. This analysis provides direct support for the proposed spatial Ca$^{2+}$ selectivity of the C- versus N-terminal lobes of CaM. Furthermore, extensive kinetic analysis strongly supports a genuine enhancement of $P_{o}$ as the underlying basis of CDF. This outcome raises novel perspectives on the potential impact of CDF at presynaptic termini.

**MATERIALS AND METHODS**

**Cell Culture and Transient Transfection**

HEK293 cells were cultured and maintained as previously described (Brody et al., 1997). To express recombinant P/Q-type channels, HEK293 cells were cotransfected with plasmids encoding: (Patil et al., 1998), allowing simpler examination of Ca$^{2+}$-dependent processes in relative isolation. The plasmid encoding the $\beta_{2}$ subunit also encoded a GFP sequence following an internal ribosomal entry site, so as to identify successfully transfected HEK293 cells by fluorescence. The RSV T antigen permits plasmid replication in HEK293 cells, thereby enhancing expression. The human $\alpha_{1A}$ (a;2:1) clone used was either an “EFa” splice variant, or an “EFb” splice variant. The exact splice background of these constructs was as follows (Soong et al., 2002; Chaudhuri et al., 2004); $\Delta 10A$ (+G); $16^{t}/17^{t}$; $17^{t}$ (~VEA). $-31^{t}$ (~NP); 37a (EFa) or 37b (EFb); 43$^{t}$/44$^{t}$/47$^{t}$.

**Electrophysiology**

2–4 d after transfection, we performed room temperature electrophysiological recordings in one of two configurations, using an Axopatch 200A amplifier (Axon Instruments).

For whole-cell recordings, the bath solution contained 150 mM tetraethylammonium methanesulfonate (TEA-MeSO$_{3}$), either 5 mM CaCl$_{2}$ or BaCl$_{2}$, 1 mM MgCl$_{2}$, and 10 mM HEPES (adjusted to pH 7.4 with TEA-OH). The internal solution contained 135 mM cesium methanesulfonate (Cs-MeSO$_{3}$), 5 mM CaCl$_{2}$, 0.5 mM EGTA, 1 mM MgCl$_{2}$, 10 mM HEPES, and freshly added 4 mM MgATP (pH 7.4, adjusted with CsOH). Electrodes were pulled with borosilicate glass capillaries (World Precision Instruments, MTW 150-F4), resulting in 1–3 MΩ resistances, before series resistance compensation of 75%. Voltage pulses were applied at 30–90 ms intervals. Currents were filtered at 2 kHz for routine rectangular step protocols, and at 5 kHz for action potential (AP) trains and tail current measurements (4-pole Bessel), and digitized at 4–5× higher frequencies. Leak and capacitance transients were subtracted by a P/8 protocol. AP voltage templates were based on recordings from the Calyx of Held (Borst et al., 1995; Patil et al., 1998).

For cell-attached single-channel currents, the bath contained 132 mM K-glutamate, 5 mM KCl, 5 mM NaCl, 3 mM MgCl$_{2}$, 2 mM EGTA, 10 mM glucose, and 20 mM HEPES (pH 7.4, adjusted with KOH). This solution zeroed the membrane potential. The pipette solution contained either 90 mM BaCl$_{2}$ or 100 mM CaCl$_{2}$, 20 mM TEA-MeSO$_{3}$, and 10 mM HEPES (pH 7.4, adjusted with...
TEA-0H). 5–20 MΩ patch pipettes were fashioned from ultra-thick-walled (2-mm outer diameter, 1.16-mm inner diameter) borosilicate glass (BF200-116-10, Sutter Instruments), and coated with Sylgard. This thick-walled glass minimized electrical noise. Voltage pulses were applied at a repetition interval of 6 s; data were sampled at 50-μs intervals and filtered at 2 kHz for Ba2+ currents and 1.25 kHz for Ca2+ currents (~3 dB, 4-pole Bessel). 

Single-Channel Analysis

Smooth functions were fitted to leak and capacity transients (P/8 protocol), and these were subtracted from single-channel records in a semiautomatic manner using custom software written in MATLAB (MathWorks). These records were then converted to idealized format using half-height criteria. Most patches contained one to two channels, with an approximately even distribution. The number of channels was judged from the maximum number of simultaneous openings (Horn, 1991). Single-channel statistics were calculated as described previously (Imredy and Yue, 1994; Patil et al., 1996; Colecraft et al., 2001). In brief, ensemble averages were normalized by the unitary current amplitude i and the number of channels N; yielding simple open probability Pi. The first latency distribution function FL was defined as the probability that a first opening occurred before time t in a voltage pulse, was calculated as

$$FL = 1 - \left(1 - FL_N\right)^{\frac{1}{N}},$$

where FL_N is the apparent first latency function for a patch containing N channels. The conditional open probability function P_{oo}(t) is defined as the probability that a channel is found open with a delay t after a channel first opens. For patches with one channel, this is calculated by time-shifting idealized records with a first opening in the first 100 ms of the test pulse, such that all first openings occur at t = 0. All such time-shifted records are then ensemble averaged and divided by i. Records with first openings after the first 100 ms of the pulse were excluded from analysis, so the calculated P_{oo}(t) would be reliable for at least 100 ms after the instant of first opening. Recall that the test pulse duration was 200–250 ms; hence, inclusion of records with first openings after the 100-ms time point would produce “edge effects” in the calculation of P_{oo}(t). In practice, the frequency of traces with first openings occurring after the 100-ms time point was exceedingly low (see FL functions in Figs. 5 and 6). With more than one channel (N > 1), the procedure was subtly modified as previously developed (Imredy and Yue, 1992; Patil et al., 1996; Colecraft et al., 2001). The basic insight for the procedure is to appropriately conceptualize the conditional expectation value for unitary current records wherein the first observed opening occurs at time t_s within the test pulse. This representation is

$$E_i(t | 1st observed opening at t_s) =$$

$$i \cdot P_o(t | a channel 1st opens at t_s) + (N - 1) \cdot i \cdot P_i(t | O during f),$$

where $E_i(t | 1st observed opening at t_s)$ is the conditional expectation in question; $P_o(t | a channel 1st opens at t_s)$ is the conditional $P_o$ for a single channel at time t given that this channel first opens at time $t_s$ and $P_i(t | O up to t_s)$ is the conditional open probability of one of the channels given that the N channels open before time $t_s$ in the test pulse. Accordingly, $P_{oo}(t)$ could be calculated as follows. For each record r (with first observed opening at time $t_r$), we initially compensated this record to produce $t_{adj}$ as given by

$$t_{adj} = t_r - (N - 1) \cdot i \cdot P_o(t | O up to t_s)$$

where $rP_o(t \mid O up to t_s)$ was calculated by (a) selecting all records lacking openings before time $t_s$ in the test pulse, (b) averaging all of these selected records, and (c) normalizing this average by N. This adjusted record $t_{adj}$ was subsequently time shifted (by $t_s$) such that its first opening then appears at $t = 0$. We ensemble averaged all such time-shifted records, and divided by i, yielding $P_{oo}(t)$ for a single channel. Regardless of the number of channels in a patch, open times were determined from all openings without stacking to a second or higher level. In multichannel patches, this procedure would in principle underestimate longer openings. However, due to the low open probability of Ca2+, in our experiments, such stacked openings were comparatively rare, and thus our open times would be insignificantly impacted.

Enhanced-opening Mechanism Simulations

The simulations in Fig. 8 were performed by numerically solving the system of differential equations that describes the enhanced-opening mechanism. Custom-written MATLAB software was used for this purpose, using standard algorithms (Patil et al., 1996) and the rate-constant parameters shown in Fig. 8. Two features merit further elaboration. First, a small fraction (~10%) of single-channel sweeps failed to exhibit any openings (“blank” sweeps). This was probably due to a small degree of cumulative voltage-dependent inactivation at the onset of test-pulse depolarizations, owing to the comparatively short interval between depolarizations in single-channel experiments (6 s). This short interval was required to obtain a sufficient number of sweeps within the typical lifetime of a cell-attached recording. By contrast, whole-cell recordings employ depolarizations every 50–90 s. To account for such cumulative inactivation in the simulation of experimentally determined $P_o$ and $FL$, the sum of all state probabilities was adjusted downward from unity, to the value of the corresponding experimentally determined $FL$t function at $t = 100$–150 ms (near the plateau). These values were 0.988 (Fig. 8 A), 0.984 (Fig. 8 B), and 0.916 (Fig. 8, C and D). Second, to explain the detailed kinetics of the $P_o$ function in Fig. 8 D, the transition from normal to facilitated modes of gating could not be modeled as a single-step transition. Instead, we adopted the simplest form of transition that depends upon the activation of multiple, independent homologous domains. More complex transitions of this form could be valid, but absent more detailed data, we have retained the simplest form of such schemes. It is assumed that while the normal mode channel is open and driving high nanomolar Ca2+ concentrations, Ca2+ binding to CaM is in rapid equilibrium with this high Ca2+, yielding B as the a fractional Ca2+ occupancy of CaM binding while the channel is open. When the channel is closed, Ca2+ is presumed to rapidly unbind from CaM. Each of four homologous domains can become permissive for facilitation (by rate constant $\alpha$) only when Ca2+ is bound to CaM; likewise, domains can only return to the nonpermissive state (by rate constant $\beta$) when Ca2+ is bound to CaM. Thus, the transitions between permissive (E) and nonpermissive (D) configurations of a homologous domain are given by the reaction scheme

$$D \xrightarrow{\alpha B \cdot P_{o(normal)}} E,$$

where $P_{o(normal)}$ is the open probability given that a channel is in the normal mode of gating. Presuming that all four homologous domains of the channel are equivalent and independent, and that they must all be permissive for the channel to become functionally facilitated (according to rate constant $\gamma$), the net transition rate from normal to facilitated modes of gating becomes $\gamma e^x$, where $e$ is the probability of a homologous domain residing in the E conformation. For the simulation in Fig. 8 D, $\alpha = 2.2 \text{ ms}^{-1}$, $\beta = 0.01 \text{ ms}^{-1}$, and $\gamma = 15 \text{ ms}^{-1}$.
Using a fast time base. Bottom, Ba^{2+} currents evoked in the same cell by the identical APW stimuli. Little facilitation or inactivation is apparent. Vertical bars, 1 nA. (B) CDF as characterized by a rectangular voltage-pulse protocol. Top, voltage pulse paradigm. To resolve CDF while minimizing CDI, test pulse depolarizations (to +5 mV) are comparatively short (50 ms). Test pulse depolarization is delivered either without a preceding voltage prepulse (gray segment), or with a brief voltage prepulse (to 5 mV) are comparatively short (50 ms). Test pulse depolarization is delivered either without a preceding voltage prepulse (gray segment), or with a brief voltage prepulse (to +20 mV). Middle, corresponding evoked Ca^{2+} currents. Without a prepulse, test pulse depolarization initially evokes rapid activation to a lower level, marked L. Following this, Ca^{2+} current (gray trace) slowly increases to a higher level H, according to CDF. When test pulse depolarization follows a voltage prepulse, Ca^{2+} current (black trace) activates immediately to the higher level, because channels have already undergone CDF in the prior prepulse. Bottom, Ba^{2+} currents evoked identically in the same cell show little indication of facilitation. Unless noted otherwise, the scale bar pertains to Ca^{2+} currents here and subsequently throughout, while Ba^{2+} currents are scaled downward by 2–3X to optimize visual comparison of kinetics. (C) Prolonged 1-s depolarization to near the peak of I_{V} relations (+10 mV) produces faster decay of Ca^{2+} versus Ba^{2+} current, indicative of CDI.

RESULTS

Macroscopic View of Ca^{2+}-dependent Facilitation and Inactivation

Fig. 1 reviews the overall features of Ca_{2,1} channel regulation by Ca^{2+}, as viewed at the level of macroscopic currents. This overview sets the stage for the ensuing focus of this study, the single-channel dissection of mechanisms underlying such regulation. During stimulation of whole-cell recombinant currents by a train of action potential waveforms (Fig. 1 A, top), the likely impact of such Ca^{2+} regulation on physiological responses becomes apparent (Fig. 1 A, middle); the initial increase in peak currents over the first several spikes reflects a Ca^{2+}-dependent facilitation process (CDF); and the subsequent decline over hundreds of milliseconds results from a Ca^{2+}-dependent inactivation process (CDI) (Lee et al., 1999; DeMaria et al., 2001). That these processes are genuinely Ca^{2+} dependent (versus voltage dependent) can be demonstrated by substituting Ba^{2+} for Ca^{2+} as the charge carrier (Fig. 1 A, bottom), whereupon peak currents remain essentially stable during an identical train. As both CDF and CDI are triggered by calmodulin (CaM) (Lee et al., 1999; DeMaria et al., 2001), the absence of regulation with Ba^{2+} fits nicely with the high Ca^{2+} over Ba^{2+} selectivity of CaM (Chao et al., 1984).

To conveniently quantify CDF and CDI, we have used rectangular voltage pulse protocols to isolate each process (DeMaria et al., 2001; Chaudhuri et al., 2004). For the more rapid process of CDF, we emphasize comparatively short test pulse depolarizations of ~50 ms (Fig. 1 B, top), a duration short enough to minimize the presence of CDI. When a test pulse is delivered in isolation, Ca^{2+} currents facilitate, as seen from a slow phase of activation (Fig. 1 B, middle, gray trace, from level L to H). Conversely, when channels become facilitated by Ca^{2+} entry during a voltage prepulse, only a rapidly activating current can be seen during the subsequent test pulse (Fig. 1 B, middle, black trace). These clear prepulse-dependent changes in both the amplitude and kinetics of test pulse currents serve as a robust measure of CDF. Consistent with this viewpoint, these changes in test pulse currents are largely absent with Ba^{2+} as charge carrier (Fig. 1 B, bottom). For the slower form of Ca^{2+}-dependent regulation (CDI), we focus upon longer step depolarizations (~1,000 ms) that permit substantial CDI to develop (Fig. 1 C), as evident from the considerably faster decay of Ca^{2+} (gray trace) versus Ba^{2+} currents (black trace). It is worth emphasizing that the CDF events described previously are still present in this Ca^{2+} trace, but are compressed within the first ~50 ms of the 1,000-ms record. Also important, the slow decay...
of Ba$^{2+}$ current reflects a slower and distinct voltage-dependent inactivation process (VDI) (Patil et al., 1998; Jones et al., 1999; Alseikhan et al., 2002; Stotz et al., 2004), whereas the large acceleration of current decay seen with Ca$^{2+}$ results from the CDI process. Hence, the large difference in Ca$^{2+}$ versus Ba$^{2+}$ current decay over hundreds of milliseconds signals the presence of CDI.

Such macroscopic indications of CDF and CDI have yielded considerable qualitative information regarding these modulatory processes (Lee et al., 1999, 2000, 2003; DeMaria et al., 2001; Soong et al., 2002; Chaudhuri et al., 2004, 2005). Nonetheless, this type of information has left unresolved several fundamental issues in regard to the elementary mechanisms that underlie these regulatory systems.

Are CDF and CDI Present at the Single-Channel Level?

A first salient question regards the purported selectivity of CaM/channel regulation for spatially different sources of Ca$^{2+}$ influx (DeMaria et al., 2001; Liang et al., 2003). Such selectivity could be tested directly at the single-channel level, where only the local (but not global) Ca$^{2+}$ signal is present by definition. Accordingly, if this principle holds true, a single CaV2.1 channel should exhibit strong CDF, but weak or absent CDI. A second open issue is whether CaV2.1 CDF represents a derepression of channel open probability ($P_o$), or a true elevation of $P_o$. Though either case would accord with macroscopic CDF behavior (Fig. 1 B), unequivocal single-channel measurement of $P_o$ would cleanly resolve the uncertainty.

Accordingly, we explicitly resolved unitary Ca$^{2+}$ currents of single recombinant CaV2.1 channels. The prepulse protocol here (Fig. 2 A, top) mirrors that described for characterization of macroscopic CDF (Fig. 1 B), except that the test pulse duration is elongated (250 ms), so as to permit detection of both CDF and CDI, if present. Inspection of the single-channel records...

**Figure 2.** Single P/Q-type channels exhibit CDF, but not CDI. (A) Unitary Ca$^{2+}$ currents during modified voltage prepulse protocol. Top, test pulse depolarizations (+20 mV) without (left) and with (right) a voltage prepulse (30 mV). Middle, single-channel records from an exemplar patch containing one channel. Dashed lines indicate the closed (top, at zero level) and open (bottom) levels of unitary current. Gray rectangle signifies period during which a sparser pattern of gating predominates. Bottom, ensemble average currents after normalization to represent $P_o$ (gray traces), as averaged from $n = 5$ patches. Smooth black curves are fitted by eye. Upper dashed line indicates zero-current level. Middle and lower dashed lines are analogous to L and H levels in Fig. 1 B. Traces show hallmarks of CDF, as present in macroscopic data (Fig. 2 B). Extreme bottom, exemplar whole-cell Ca$^{2+}$ current evoked by +10-mV depolarization, illustrating clearly detectable CDI over the time period observed in single-channel recordings. Absence of such decay in ensemble average currents indicates lack of CDI at the single-channel level. (B) Unitary Ba$^{2+}$ currents observed under an identical protocol; identical format as in A. Middle, exemplar single-channel records from a patch containing one P/Q-type channel. Calibration bar identical to that in A. Bottom, ensemble average currents normalized to $P_o$, averaged from $n = 5$ patches. Note the absence of a sparser pattern of gating during the corresponding period denoted by a gray rectangle.
(Fig. 2 A, middle) readily reveals stochastic millisecond gating, with small unitary currents of $i \sim 0.4 \, \text{pA}$. The ensemble average of such activity (Fig. 2 A, bottom, from \( n = 5 \) patches) clearly resolves prepulse-dependent changes that are characteristic of robust CDF. Specifically, in the absence of a prepulse, channels initially feature rapid opening to a low $P_o \sim 0.04$ (Fig. 2 A, bottom left), followed by a slow transition to a higher $P_o \sim 0.09$. However, after prepulse depolarization, channels open immediately to the higher $P_o \sim 0.09$ (Fig. 2 A, bottom right). The overall similarity to macroscopic CDF events is unmistakable (compare Fig. 1 B, middle). By contrast, CDI is absent in these same patches; once achieved, the $P_o \sim 0.09$ is essentially maintained for the entire duration of the 250-ms stimulus (Fig. 2 A, bottom, left and right). For reference, a comparable macroscopic current record exhibits an unmistakable decline over the same time frame (Fig. 2 A, far bottom right). These results directly demonstrate that CaM-dependent processes, as triggered by the C- and N-terminal lobes of CaM, are indeed differentially sensitive to local versus global Ca$^{2+}$ signals.

The direct resolution of $P_o$ also enables us to determine whether CDF merely relieves an initial suppression of basal $P_o$ or actually boosts $P_o$ over a standard level. To distinguish between these contrasting scenarios, we determined the reference $P_o$ seen with Ba$^{2+}$ as the charge carrier (Fig. 2 B). Since CDF should be absent with Ba$^{2+}$, the $P_o$ determined here would provide a baseline against which to judge the actions of CDF. Fig. 2 B shows the single-channel activity with Ba$^{2+}$ as charge carrier (middle), evoked with a directly comparable prepulse protocol (top). Unitary current amplitudes are larger ($i \sim 0.9 \, \text{pA}$), and the ensemble averages (bottom, $n = 5$ patches) are identical regardless of a prepulse (Colecraft et al., 2001), confirming the absence of appreciable facilitation. More importantly, the basal $P_o$ reaches $\sim 0.04$, which is clearly smaller than the Ca$^{2+}$-facilitated level (compare Fig. 2 A, bottom). Hence, CDF reflects an actual boost in $P_o$ compared with the basal state, rather than derepression of an initially inhibited configuration. Further evidence that Ba$^{2+}$ currents represent a bona fide reference baseline for Ca$^{2+}$ activity is presented later in Fig. 6.

**Contrasting CDF Mechanisms: Enhanced Steady-State Opening versus Accelerated Activation**

Beyond the fundamental concerns of spatial Ca$^{2+}$ selectivity of CDF versus CDI, and genuine enhancement of $P_o$ by CDF, more in-depth analysis of the single-channel activity (Fig. 2, A and B, middle) could reveal the explicit gating mechanism that underlies the enhancement of $P_o$. To focus this analysis, we first articulate the two alternative gating mechanisms that could potentially explain CDF (Fig. 3 A). The enhanced opening hypothesis predicts that nonfacilitated channels open into a “normal” mode of gating, characterized by a lower steady-state $P_o$ as seen with Ba$^{2+}$ (Fig. 3 A, left, white states). CDF then results from a CaM-mediated transition to a ‘facilitated’ mode of gating, featuring a higher steady-state $P_o$ (Fig. 3 A, left, gray states). In Fig. 2 B (left), cartoons illustrate the single-channel patterns corresponding to this proposed mechanism. Without a prepulse (top), a channel opens without much delay, but initially shows the sparser opening pattern of the normal mode. Upon a discrete transition to the enhanced opening mode (top, arrow), the channel adopts a more active opening pattern. After a prepulse (bottom), a channel often starts the ensuing test pulse within the facilitated opening mode. Single-channel activity thereby shows rapid opening directly into the enhanced-opening pattern, which is maintained throughout the test pulse. By contrast, the accelerated activation hypothesis (Fig. 3 A, right) predicts that channels in the facilitated mode first open faster than those in the normal mode, but that channels in either mode maintain similar to identical open probabilities subsequent to first opening (Fig. 3 A and B, right). Mechanisms of this type have been proposed to explain CDF as observed in native P-type Ca$^{2+}$ channel in the calyx of Held (Borst and Sakmann, 1998). Fig. 3 B emphasizes how these different single-channel gating properties would nonetheless elaborate macroscopic currents with closely similar profiles. Moreover, a mixture of these two mechanisms could also support the same macroscopic behavior.

With this view of potential gating schemes in mind, we devise customized means of determining the extent to which each mechanism underlies CDF. Protocols and analysis at both the whole-cell and single-channel levels can provide independent lines of evidence favoring either or both mechanisms, as follows.

**Macroscopic Tail Current Analysis of CDF**

A first strategy to elucidate the underlying gating mechanisms for CDF concerns whole-cell tail current protocols, which would produce voltage-dependent activation curves as hypothesized in Fig. 3 C. In this protocol, currents are activated by a brief (20-ms) voltage step to a variable potential ($V_{\text{step}}$), followed by repolarization to an invariant tail potential ($V_{\text{tail}}$). Given this setup, rapidly decaying “tail currents” will be produced during the repolarization, the peak amplitude of which ($I_{\text{tail}}$) will be given by

$$I_{\text{tail}} = N \cdot P_o(V_{\text{step}}) \cdot g \cdot (V_{\text{tail}} - V_{\text{rev}}),$$

where $N$ is the number of channels in the cell, $P_o(V_{\text{step}})$ is the open probability reached at the end of the 20-ms voltage step, $g$ is the single-channel conductance, and $V_{\text{rev}}$ is the reversal potential. Because all factors are held constant, except for $V_{\text{step}}$, normalization of the tail current...
yields a relative measure of $P_0(V_{step})$. Importantly, the $P_0(V_{step})$ that we measure in this way represents a type of weighted average of the voltage-dependent activation profiles for normal and facilitated modes of gating. This experimental $P_0$ metric thereby predicts different outcomes for enhanced opening versus accelerated activation mechanisms (Fig. 3 A).

For the enhanced opening mechanism, the steady-state $P_0-V$ relation for the facilitated gating mode (Fig. 3 C, left, gray curve) would be larger in amplitude, and possibly leftward shifted, compared with the relation for the normal mode (black dashed curve). Because the voltage step furnishes ample time for channels to reach steady state in either gating mode of this scenario, the experimental activation curve determined from tail currents (Fig. 3 C, left, black solid curve) should reflect a true weighted average of the steady-state relations for each mode in isolation (gray and black dashed curves), where the weighting is by the fraction of channels in each mode at the end of the step depolarization. Because the fraction of facilitated channels at the end of the step ($F_{fac}$) will be proportional to the Ca$^{2+}$ influx driving CDF, and Ca$^{2+}$ entry itself features a bell-shaped voltage dependence, it follows that $F_{fac}$ should also demonstrate a like biphasic profile. The enhanced opening mechanism predicts a hump-shaped relative $P_0$ curve as determined from tail current measurements. This distinctive shape arises from the smaller plateau level of steady-state $P_0$ curves for the normal (black dashed curve) versus facilitated modes of gating (gray curve), the weighted average of which produces the measured relative $P_0$ curve (solid black). The relevant weighting factor is $F_{fac}$, the fraction of channels in the facilitated mode at the end of the test depolarization just before capturing tail currents. The accelerated-activation mechanism predicts a measured relative $P_0$ curve (solid black) that lacks an overshoot hump. For short 20-ms depolarizations before tail current measurement, the corresponding $P_0$ curves for normal (gray) and facilitated modes of gating (black dashed) are simply voltage shifted with respect to each other, such that their weighted average cannot produce an overshoot. (D and E) Expected single-channel outcomes for enhanced-opening (left) and accelerated-opening (right) mechanisms.

Figure 3. Potential elementary mechanisms underlying macroscopic CDF. (A) State diagram representation of mechanisms. For the enhanced-opening mechanism (left), the normal mode of gating (states within white box) features rapid activation with a lower $P_0$ after first opening, whereas the facilitated mode (states within gray box) supports nearly identical activation with a higher $P_0$ after first opening. Ca$^{2+}$ binding to CaM drives the transition from normal to facilitated modes, thereby producing CDF. C represents a closed conformation, and O represents an open conformation. For the accelerated-activation mechanism (right), the normal mode exhibits a delayed time to first opening, but an appreciable $P_0$, whereas the facilitated mode supports fast first opening, but the same $P_0$, thereafter. The facilitated mode sports fast first opening, but the same $P_0$, thereafter. The facilitated mode sports fast first opening, but the same $P_0$, thereafter.

(B) Cartoons of expected single-channel activity for the enhanced-opening (left) and accelerated-activation (right) mechanisms. Top, anticipated activity for test pulse depolarization without prepulse. Bottom, expected activity for test pulse with prepulse. Labels denote gating mode associated with indicated segments of activity; arrow marks transition between modes. Either scenario (left or right) would give rise to the well-studied macroscopic profile of CDF, schematized in the center of the panel. (C) Expected distinctions between the two mechanisms, when probed with macroscopic tail current analysis (e.g., Fig. 4). The enhanced-opening mechanism (left) predicts a hump-shaped relative $P_0$ curve as determined from tail current measurements. This distinctive shape arises from the smaller plateau level of steady-state $P_0$ curves for the normal (black dashed curve) versus facilitated modes of gating (gray curve), the weighted average of which produces the measured relative $P_0$ curve (solid black). The relevant weighting factor is $F_{fac}$, the fraction of channels in the facilitated mode at the end of the test depolarization just before capturing tail currents. The accelerated-activation mechanism predicts a measured relative $P_0$ curve (solid black) that lacks an overshoot hump. For short 20-ms depolarizations before tail current measurement, the corresponding $P_0$ curves for normal (gray) and facilitated modes of gating (black dashed) are simply voltage shifted with respect to each other, such that their weighted average cannot produce an overshoot. (D and E) Expected single-channel outcomes for enhanced-opening (left) and accelerated-opening (right) mechanisms.

$FL$ is the probability that a first opening occurs before time $t$ in the test pulse depolarization (D), whereas $P_{oo}$ is the probability of finding a channel open with a delay $t$ after a channel is known to have had a first opening (E). The enhanced-opening mechanism predicts no prepulse dependence of $FL$ (D, left), but a characteristic prepulse dependence of $P_{oo}$ (E, left). The accelerated-activation mechanism will show just the opposite profile (D and E, right) (see text for further details).
a distinctive experimental activation curve with an “overshoot hump” (Fig. 3 C, left, solid black curve), which should be centered around voltages that maximize Ca\(^{2+}\) influx during the step.

For the accelerated-activation mechanism, both normal and facilitated modes support similar \(P_o-V\) relations at steady state, as these modes are defined by comparable \(P_o\) values after initial opening (Fig. 3 C, right, gray curve). However, though facilitated channels would activate quickly enough to reach their steady-state profile within the 20-ms test pulse, slower-to-activate channels within the normal mode of this mechanism would not achieve steady state, yielding an effective activation curve that would appear right shifted (Fig. 3 C, right, black dashed curve). Curves for the two modes would likely exhibit the same plateau level at saturating depolarization, because very strong depolarization would probably produce sufficient acceleration of normal channel activation to permit attainment of steady-state gating. Accordingly, by using the anticipated \(F_{\text{fac}}\) function (Fig. 3 C, right, bottom), the weighted average of these two \(P_o-V\) relations (Fig. 3 C, right, gray and black-dashed curves) predicts an experimental activation curve that lacks a tell-tale hump (Fig. 3 C, right, black solid curve).

Fig. 4 summarizes the results for the actual tail current experiments. With Ca\(^{2+}\) as charge carrier, tail current amplitudes are indeed maximized by an intermediate step depolarization to \(~40\) mV (Fig. 4 A, middle). With either more hyperpolarized (20 mV) or depolarized (120 mV) voltage steps, tail currents are comparatively smaller. By contrast, corresponding Ba\(^{2+}\) tail currents increase monotonically with strengthened depolarization (Fig. 4 A, bottom), as expected for channels trapped within a normal gating mode. Activation curves, averaged from \(n=5\) cells after normalizing to responses for the 120-mV depolarization, confirm a clear overshoot in the Ca\(^{2+}\) activation curve (Fig. 4 B, solid circles), which is absent with Ba\(^{2+}\) (open circles). Subtracting these activation curves reveals a bell-shaped curve (Fig. 4 B, bottom), analogous to that expected for \(F_{\text{fac}}\) (Fig. 3 C, bottom). These results suggest that an enhanced opening mechanism is at least partly responsible for CDF. However, this type of data does not exclude the additional presence of accelerated activation, nor is the interpretation favoring enhanced opening entirely independent of assumptions regarding the behavior of \(P_o-V\) relations for the different gating modes.

In Depth Single-Channel Analysis of CDF

Rigorous statistical analysis of single-channel activity promises a more definitive dissection of CDF mechanisms. Direct visual inspection of unitary currents provides an initial indication of the underlying CDF mechanism. Indeed, exemplar records (Fig. 2 A) hint at a period of sparser opening near the onset of voltage steps without a prepulse (Fig. 2 A, gray bar period), which is nearly absent following a prepulse. These records also fail to suggest marked prepulse-dependent differences in the time to first opening (first latencies). These visual trends thereby give the impression of an enhanced opening mechanism. More rigorously, the single-channel data permit accumulation of histograms describing first latency probability (\(FL\)) and conditional open probability (\(P_{o|L}\)) functions, as theoretically portrayed in Fig. 3 (D and E). These two metrics are ideally poised to distinguish among CDF mechanisms, as follows. \(FL(t)\) quantifies the probability that the first channel opening occurs before a time \(t\) within the test pulse, and fully incorporates the kinetics of initial channel opening occurring before the most extreme depolarizations shown. Ba\(^{2+}\) from accounts for the surface charge shift between Ca\(^{2+}\) ages have been shifted 12 mV in the depolarizing direction, to fits to Ca\(^{2+}\) currents. Smooth curve fits by eye. Bottom, difference of smooth 392 Mechanisms of Facilitation in Ca\(_{2.1}\).
activation (Aldrich and Stevens, 1983). $P_{oo}(t)$, on the other hand, is the conditional open probability that a channel will be found open with a delay $t$ after a first opening; this measure thereby specifies aggregate channel gating after the initial activation event. Together, these two functions constrain the overall gating characteristics of a channel (Sigworth, 1981; Aldrich and Stevens, 1983; Imredy and Yue, 1994; Patil et al., 1996; Colecraft et al., 2001) and project very different outcomes for the contrasting CDF mechanisms. For the enhanced opening scheme, $FL$ curves derived from test pulses with or without a prepulse should be nearly identical (Fig. 3 D, left), because initial gating is not appreciably different in normal versus facilitated gating modes. However, the accelerated activation mechanism predicts faster rising $FL$ curves following a prepulse, since facilitated channels would first activate faster in this scenario (Fig. 3 D, right). With the $P_{oo}$ function, the situation is reversed. The enhanced opening hypothesis posits that, during isolated test pulses, the conditional $P_{oo}$ following first opening would initially feature a small value ($P_{oo} \sim 0.04$), which would then transition to a larger level ($P_{oo} \sim 0.09$). This effect should be visible as a dip in the $P_{oo}$ curve before reaching a plateau at longer $t$ (Fig. 3 E, left). For test pulses that follow a prepulse, the dip should be absent, as channels open directly to the $P_{oo} \sim 0.09$ level. Conversely, in the accelerated activation hypothesis, no difference exists in the conditional $P_{oo}$ subsequent to first opening, so $P_{oo}$ curves from test pulses should be almost identical without regard to a preceding prepulse (Fig. 3 E, right).

Turning to our data, in depth single-channel analysis furnishes definitive evidence favoring the predominance of an enhanced opening mechanism for CDF (Fig. 5). First, $FL$ distributions for $Ca^{2+}$ appear essentially identical without regard to the presence of prepulse depolarization (Fig. 5 A, left, average from $n = 5$ patches), contradicting an accelerated activation model. In $Ba^{2+}$, $FL$ curves also appear superimposed (Fig. 5 A, right, average from $n = 4$ patches). Second, $P_{oo}$ curves with $Ca^{2+}$ do indeed show prepulse-dependent changes characteristic of the enhanced opening mechanism. Without a prepulse, the $P_{oo}$ curve dips to a low point ($\sim 0.04$) before transitioning to a higher plateau with $P_{oo} \sim 0.09$ (Fig. 5 B, left, gray curve average of $n = 5$ patches). With a prepulse, the $P_{oo}$ rapidly achieves the higher level without evidence of a dip (black curve average of $n = 5$ patches). These prepulse-dependent differences are confirmed by calculating the difference between $P_{oo}$ curves (Fig. 5 C, top left), which reveals a roughly single-exponential decay, with time constant $\sim 20$ ms. By integrating this difference, we emphasize further the significance of the difference curve, as the integrated relation flattens out at a distinctly negative value (Fig. 5 C, bottom left). Third, these prepulse-dependent differences in $P_{oo}$ clearly reflect CDF, because of their complete absence in the case of $Ba^{2+}$ activity (Fig. 5 B, right). Here, regardless of prepulse, the $P_{oo}$ functions decay immediately to the same lower level of $\sim 0.04$, without indication of a dip. In all, the nearly exact correspondence of these actual $FL$ and $P_{oo}$ profiles to those predicted for the enhanced opening scheme (Figs. 3, D and E, left) furnishes direct support for this mechanism of CDF.

**Figure 5.** In depth single-channel analysis supports a nearly exclusive enhanced-opening mechanism. For all panels, the left column pertains to mean statistical profiles for unitary $Ca^{2+}$ currents through $Ca_{V2.1}$ channels (EFb splice variant), whereas the right column concerns mean statistics for unitary $Ba^{2+}$ currents through the same type of channels. (A) $FL$ functions measured with (black curve) and without (gray curve) a preceding prepulse. There is no appreciable prepulse dependence. $Ca^{2+}$ curves averaged from $n = 5$ patches; $Ba^{2+}$ curves from a different $n = 4$ patches. (B) $P_{oo}$ functions measured with (black curve) and without (gray curve) a prepulse. Smooth curves fit by eye. The $Ca^{2+}$ relation obtained without a prepulse exhibits the characteristic “dip” expected for an enhanced opening mechanism. $Ca^{2+}$ data averaged from $n = 5$ patches. $Ba^{2+}$ data averaged from a different $n = 5$ patches. (C) Top, difference of mean $P_{oo}$ relations in B (no prepulse $P_{oo}$– prepulse $P_{oo}$). Bottom, time integral of difference relations above (ms units, shown at $20 \times$ amplification). Top left, difference relation for $Ca^{2+}$ currents fit by eye with single exponential function with a time constant of 20 ms.
contexts (Lipscombe et al., 2002; Lipscombe and Castiglioni, 2003). In this regard, another worthy application of single-channel analysis concerns the behavior of a fascinating EFb splice variant of CaV2.1, wherein alternative splicing at a single exon ablates CDF of these channels (Chaudhuri et al., 2004; Chaudhuri et al., 2005). All experiments described thus far in this study have used the EFa splice variant of CaV2.1, which is permissive for CDF. An important question is whether EFb channels are trapped within the normal versus facilitated mode of gating. This question remains open, as sequestration within either mode would explain the observed insensitivity of macroscopic Ca\(^{2+}\) currents to prepulse depolarization.

Fig. 6 summarizes single-channel data, which definitively indicate that EFb channels are restricted to the normal mode of gating, as portrayed in the enhanced opening scheme of Fig. 3 A (left). Regardless of prepulse, unitary Ca\(^{2+}\) currents from this splice variant exhibit a sparser gating pattern (Fig. 6 A, middle), and the ensemble averages invariably demonstrate a lower overall \(P_{\text{oo}}\) of \(\sim 0.04\) (Fig. 6 A, bottom, averaged from \(n = 5\) patches). Moreover, both \(FL\) and \(P_{\text{oo}}\) functions exhibit strict prepulse insensitivity (Fig. 6, B and C), and the \(P_{\text{oo}}\) relation decays directly to a plateau value of \(\sim 0.04\). These two relations quantitatively recapitulate the fingerprint of isolated normal mode gating, as gauged by the corresponding metrics for unitary Ba\(^{2+}\) currents of EFa channels (Fig. 2 B and Fig. 5).

This entrapment of EFb channels (and of EFa channels with Ba\(^{2+}\)) motivates a final dissection of CDF mechanisms using more traditional methods. More precisely, captivity within a single gating mode simplifies the application of classical open-duration histogram
The accelerated-activation mechanism would predict only an increase in amplitude, without change in longevity.

Physiological Implications of Enhanced Opening versus Accelerated Activation Mechanisms

Having established the enhanced opening mechanism, we explore a correlative physiological implication of CDF, using our new mechanistic insights to reexamine the macroscopic Ca\(^{2+}\) currents evoked by action-potential waveform (APW) trains (Fig. 1 A). The spike-to-spike increases of Ca\(^{2+}\) current amplitude reflects a net increase in the product of two multiplicative factors: the probability of observing a channel open at least once during a particular stimulus (\(P_{\text{gate}}\)), and the conditional \(P_{\text{fl}}\) given that at least one opening does occur (\(P_{\text{oligate}}\)). The enhanced-opening mechanism predicts that repetitive spike activation would leave \(P_{\text{gate}}\) largely unchanged, because \(F_{\text{l}}\) is little changed in this kinetic framework. Instead, the increased current would mainly reflect progressive augmentation and prolongation of \(P_{\text{oligate}}\), as driven by conversion of channels to a facilitated mode with higher characteristic \(P_{\text{fl}}\) but similar activation kinetics. Hence, macroscopic current responses should not only become larger, but longer lasting. By contrast, an accelerated-activation mechanism would predict only a boost in \(P_{\text{gate}}\), reflective of changes in \(F_{\text{l}}\). Because gating after first opening is unchanged with facilitation in this context, \(P_{\text{oligate}}\) would remain largely unchanged. Accordingly, macroscopic currents should increase in amplitude alone, without alteration in waveform shape. We test for these predictions by normalizing the peak amplitudes of currents evoked by the first stimulus to those seen for the largest subsequent response during an APW train. With Ba\(^{2+}\) as charge carrier, there is an insignificant increase in current during trains (Fig. 1 A, bottom; 103 ± 1% increase, \(n = 3\) cells, n.s.). Moreover, first and maximal Ba\(^{2+}\) responses show the identical waveform shape after normalization (Fig. 7 A, bottom). For quantification of normalized waveform duration, we measure the difference in time at which waveforms relax to half-maximal amplitude (\(\Delta t_{1/2} = 13 ± 13\ \mu s, n = 3\) cells, n.s.). By contrast, Ca\(^{2+}\) responses for EF\(_a\) channels not only showed significant increases in amplitude (Fig. 1 A, 122 ± 5%, \(n = 3\) cells, \(P < 0.05\) compared with Ba\(^{2+}\)), but also substantial waveform prolongation (Fig. 7 B, \(\Delta t_{1/2} = 120 ± 23\ \mu s, n = 3\) cells, \(P < 0.05\).

Figure 7. Resolving dual dimensions of APW-induced current augmentation, as predicted by the enhanced-opening mechanism of CDF. (A) Alignment of first (dashed black curve) and maximal Ca\(^{2+}\) current responses (solid black curve) from APW train experiment in Fig. 1 A. Upward scaling of the first response, to match the amplitude of the maximal response, yields a scaled waveform (gray curve) that superimposes the maximal response (black solid curve). Hence, CDF yields Ca\(^{2+}\) responses that are both larger and longer lasting, as predicted by the enhanced opening mechanism. The accelerated-activation mechanism would seemingly complicate interpretation of open state is potentially slower in the facilitated versus normal gating mode of the enhanced opening scheme, open durations might be (a) identically short for channels imprisoned within the normal mode, and (b) prolonged for channels strongly enriched for facilitated openings. Alternatively, the accelerated activation model would predict similar open durations for both instances, as gating is presumed similar after first opening. Support for the first prediction comes from the indistinguishably brief open durations for Ba\(^{2+}\) currents through EF\(_a\) channels (Fig. 6 D) and Ca\(^{2+}\) currents through EF\(_b\) channels (Fig. 6 E). As expected, neither of these configurations exhibit detectable prepulse dependence, because of restriction to the normal gating mode. As for the second prediction, the mode switching of unitary Ca\(^{2+}\) currents through EF\(_a\) channels would seemingly complicate interpretation of open times as cleanly representative of either normal or facilitated configurations in seclusion. Further consideration would, however, suggest that although channels may spend a visible span of time within the normal gating mode (Fig. 2 A, gray bar period, no prepulse case), the numerically few openings that occur within this sparse pattern of activity will be far outweighed by the vast number of openings within the presumed facilitated context. Hence, even without prepulse, the open duration histogram should very nearly reflect properties of the facilitated gating mode viewed in isolation. Consistent with this view, open durations for Ca\(^{2+}\) currents through EF\(_a\) channels are essentially independent of prepulse depolarization, and elongated relative to the other configurations (Fig. 6 F). Superposition of open time histograms for the various contexts explicitly emphasizes these findings (Fig. 6 G). For clarity, only data following prepulse depolarization are shown in the latter overlay. Overall, this open duration analysis supports an enhanced opening mechanism for CDF, along with confinement of channels to the normal gating mode (EF\(_a\) carrying Ba\(^{2+}\) currents, or EF\(_b\) channels).
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This bidimensional augmentation of spike responses (amplitude and duration), as suggested by the enhanced opening mechanism, may entail significant biological consequences, given the power-law relationship between presynaptic Ca\textsuperscript{2+} influx and neurotransmitter release (Dodge and Rahamimoff, 1967), as well as the exquisite spatio-temporal coupling of Ca\textsuperscript{2+} sources to vesicle release (Zucker and Regehr, 2002). For example, prolongation of spike-evoked Ca\textsuperscript{2+} responses by 100–200 \(\mu\)s, even without appreciable change in peak amplitude, can enhance post-synaptic EPSCs by two to threefold (Sabatini and Regehr, 1997). More broadly, spike-dependent changes in Ca\textsuperscript{2+} currents have also been observed in the context of dynamic G-protein inhibition of Ca\textsubscript{v}2 channels (Brody et al., 1997; Park and Dunlap, 1998). The activity-dependent relief from inhibition for certain Ca\textsubscript{v}2.2 channels is akin to an enhanced opening mechanism (Colecraft et al., 2001) and supports spike-dependent enhancement of current amplitude and duration. By contrast, the analogous reversal of G-protein inhibition in Ca\textsubscript{v}2.1 channels involves an accelerated activation scheme (Colecraft et al., 2001), and spike-dependent augmentation of current transpires by changes in amplitude alone.

DISCUSSION

This paper reports on the first extensive single-channel analysis of Ca\textsubscript{v}2.1 channels permeated with the physiological charge carrier Ca\textsuperscript{2+}. These recordings resolve fundamental mechanistic unknowns regarding the CaM-mediated Ca\textsuperscript{2+} feedback of these channels. First, we provide direct evidence that CDF (triggered by the C-lobe of CaM) responds to a local Ca\textsuperscript{2+} signal, whereas CDI (triggered by the N-lobe of CaM) requires a global Ca\textsuperscript{2+} signal. This demonstration substantiates key features of a general rule of spatial Ca\textsuperscript{2+} preference concerning the C- versus N-lobe of CaM, as seen for CaM regulation across the family of Ca\textsubscript{v}1-2 channels (Liang et al., 2003). Second, independent lines of evidence from customized whole-cell and single-channel experiments demonstrate that CDF results from a genuine enhancement of channel opening probability over a basal level, rather than derepression of channel \(P_o\) or acceleration of activation kinetics. Splice variation at an EF-hand locus of Ca\textsubscript{v}2.1 channels can restrict conformations to a normal gating mode, which supports the basal level of activity. Third, the enhanced-opening mechanism may support increases in both the amplitude and duration of Ca\textsuperscript{2+} currents evoked during repetitive physiological stimuli. This section contextualizes these new data within the body of prior single-channel experiments, broadens the neurobiological implications of the spatial Ca\textsuperscript{2+} selectivity of CaM regulatory mechanisms revealed here, and explores the generality of the enhanced opening mechanism of CDF.
An important caveat relating to the majority of single-channel data in the literature concerns the use of ~100 mM charge carrier to facilitate resolution of unitary currents. This limitation also pertains to the present study and merits some caution in quantitative extrapolation to the physiological context where ~2 mM Ca2+ is the charge carrier. On the other hand, whole-cell records using near physiological Ca2+ do exhibit similar phenomena to those produced by single-channel activity (compare Fig. 1 B and Fig. 2). This latter concordance argues that qualitative extrapolations between the two regimes are warranted.

Neurobiological Implications of the Spatial Ca2+ Selectivity of CaM-mediated Feedback

Over the past several years, whole-cell experiments probing CaM/Ca2+ channel regulation have hinted at the existence of an intriguing design principle for the spatial Ca2+ selectivity of the lobes of CaM (Liang et al., 2003). Across the CaV1-2 channel family, different forms of CaM/channel regulation can be initiated by Ca2+ binding to one lobe of CaM or the other (Peterson et al., 1999; DeMaria et al., 2001; Lee et al., 2003; Liang et al., 2003). In the extreme, two different regulatory processes can be observed within a single channel type (e.g., CDI and CDF of CaV2.1; or slow and fast CDI in CaV1.3), with each process selectively triggered by Ca2+ binding to a different lobe of CaM (DeMaria et al., 2001; Yang et al., 2006). Remarkably, whenever Ca2+ binding to the C-terminal lobe of CaM triggers regulation, this regulation is insensitive to strong Ca2+ buffering, even by several millimolar concentrations of the rapid chelator BAPTA (Zuhlke and Reuter, 1998; DeMaria et al., 2001; Soong et al., 2002; Chaudhuri et al., 2004, 2005; Yang et al., 2006). Such buffering would zero Ca2+ everywhere except within the very local nanodomain of Ca2+ channels (Augustine et al., 2003). Moreover, the strength of such regulation is largely independent of current amplitude (Peterson et al., 1999; Soong et al., 2002; Chaudhuri et al., 2004, 2005; Yang et al., 2006), which serves as an indicator of the global Ca2+ signal. The indirect inference from these experiments is that the C-terminal lobe of CaM responds preferentially to local Ca2+ signals. The only known exception to this trend is the CDF (mediated by the C-terminal lobe) for a particular splice variant of P/Q-type channels (EFb with exon 47 present), which exhibits a partial global Ca2+ preference (Chaudhuri et al., 2004). By overall contrast, whenever Ca2+ binding to the N-terminal lobe of CaM initiates channel modulation, this modulation is strongly sensitive to Ca2+ buffering (Lee et al., 2000; DeMaria et al., 2001; Soong et al., 2002; Liang et al., 2003), and the strength of this modulation is clearly correlated with whole-cell current amplitudes (Soong et al., 2002; Yang et al., 2006). Hence, it would appear that the N-terminal lobe of CaM is selective for global Ca2+ signals, even though the relevant CaM molecule appears constitutively bound to channels within the nanodomain. That this pattern holds true across multiple CaV1-2 channels suggests that this pattern of spatial Ca2+ selectivity reflects a general principle (Liang et al., 2003).

The single-channel experiments in this study (Fig. 2 A) furnish the most direct evidence to date for this pattern of spatial Ca2+ preference. By definition, a single channel can only produce a local Ca2+ signal, but not a global Ca2+ signal. Hence, the preservation of CDF (driven by the C-lobe of CaM) and absence of CDI (induced by the N-lobe) within single CaV2.1 channels directly demonstrates the distinctive spatial Ca2+ preferences of the lobes of CaM. It might be questioned whether the lack of single-channel CDI is trivially produced by depolarizing to a potential (+20 mV) that is nonpermissive for CDI, considering that the CDI of macroscopic currents is only apparent over a certain voltage range (Chaudhuri et al., 2004). However, macroscopic current data also indicate that the permissive voltages for CDF and CDI are coincident (Chaudhuri et al., 2004). Hence, the presence of strong CDF in single-channel patches confirms that the lack of CDI is not trivially produced by an inappropriate test potential, and that the mechanistic deductions concerning spatial Ca2+ selectivity hold true. An important contrasting control for the lack of CDI in single CaV2.1 channels is the clear CDI of CaV1.2 channels observed at the single-channel level (Yue et al., 1990; Imredy and Yue, 1994). Here, the CDI is triggered by Ca2+ binding primarily to the C-terminal lobe of CaM, and would be expected to be responsive to local Ca2+ signals (Peterson et al., 1999). Of further importance, the intracellular Ca2+ buffering status in these single-channel experiments is completely unperturbed from the native configuration, as no intracellular dialysis occurs in the on-cell patch-clamp configuration that we used (Hamill et al., 1981). Hence, the proposed lobe-specific pattern of spatial Ca2+ selectivities is relevant to native cells, not only those in which whole-cell dialysis of the intracellular milieu may have inadvertently accentuated such a pattern. Experiments that probe unitary Ca2+ currents through other CaV1-2 channels can further extend the generality of CaM lobe-specific selectivities for spatial Ca2+ signals.

Given the strong evidence for lobe-specific spatial Ca2+ preference in CaV2.1 channels, it is worth considering obvious neurobiological implications of this selectivity (Evans and Zamponi, 2006). In particular, as CaV2.1 channels comprise the dominant Ca2+ trigger of synaptic transmission within the central nervous system (Wheeler et al., 1994; Dunlap et al., 1995; Wheeler and Tsien, 1999), their dynamic regulation by Ca2+ may impact neuronal information transfer via short-term synaptic plasticity (Tsodyks and Markram, 1997; Tsodyks...
Figure 8. Explicit kinetic simulations of the enhanced-opening mechanism furnish a global quantitative explanation for multiple single-channel datasets. (A) Simulations of unitary Ba$^{2+}$ activity through channels bearing the EF$a$ splice variant. Far left, kinetic layout and rate-constant parameters for transitions within the normal mode of gating at a step potential of +20 mV (white rectangle). Numerical values are in units of ms$^{-1}$. Parameters and states shown in black are best determined, being doubly constrained by $P_o$ and $P_{oo}$ functions. Those displayed in gray impart subtle improvements to the overall fit, but are less well constrained. Mean experimental $P_o$ (center left), $P_{oo}$ (center right), and FL (far right) are reproduced from earlier data figures as gray traces (prepulse data only). The initial conditions of the $P_o$ and FL simulations featured 20% of available channels in the leftmost closed state, and 80% of available channels in the immediately adjacent closed state. Superimposed upon these plots are the kinetic simulations of the enhanced-opening mechanism (black curves), with parameters on the far left. (B) Simulations of unitary Ca$^{2+}$ activity through EF$b$ channels, using identical parameters as in A, except that the initial conditions of $P_o$ and FL simulations featured 15% of available channels in the leftmost closed state, and 85% of available channels in the immediately adjacent closed state. Format as in A. (C) Simulations of unitary Ca$^{2+}$ activity through EF$a$ channels, after a prepulse. Format as in A, except that these data help constrain the facilitated mode parameters (gray rectangle) in relative isolation. The main difference of the facilitated mode, as compared with the normal mode (A and B), concerns only the transitions between the final closed and open states (shown in bold). Subtle differences in the exit transitions from the two leftmost closed states afford modest improvements of the fit to FL data, while voltage inactivation is left unchanged. The initial conditions of $P_o$ and FL simulations featured 15% of available channels in the leftmost closed state of the facilitated mode, and 85% of available channels in the immediately adjacent closed state. (D) Simulations of unitary Ca$^{2+}$ activity through EF$a$ channels, without a prepulse. Format as in A, except that these data help constrain the facilitated mode parameters (gray rectangle) in relative isolation. The main difference of the facilitated mode, as compared with the normal mode (A and B), concerns only the transitions between the final closed and open states (shown in bold). Subtle differences in the exit transitions from the two leftmost closed states afford modest improvements of the fit to FL data, while voltage inactivation is left unchanged. The initial conditions of $P_o$ and FL simulations featured 15% of available channels in the leftmost closed state of the facilitated mode, and 85% of available channels in the immediately adjacent closed state. (E) Simulations of unitary Ca$^{2+}$ activity through EF$b$ channels, without a prepulse. Format as in A. The facilitated mode parameters are maintained exactly as in C. The normal mode parameters are identical to those used above (A and B), except for minor differences in the exit rates for the two leftmost closed states. The latter differences yield small improvements to the fit of FL. For $P_o$, $P_{oo}$, and FL simulations, the initial fraction of available channels in the facilitated mode was 35%, with 65% in the normal mode. The initial fraction of channels in the facilitated mode probably has to do with the comparatively short 6-s repetition interval used in single-channel recordings, producing some cumulative CDF. The recovery time course from CDF is ~0.5 s (DeMaria et al., 2001). Additionally, for $P_o$ and FL simulations, the initial configuration was for 15% of channels in either mode to reside in the leftmost closed state, and for the remaining 85% of channels in either mode to reside in the immediately adjacent closed state. The transition from normal to facilitated modes occurs via a simple cooperative transition, as detailed in the Materials and methods.
et al., 1998; Zucker and Regehr, 2002; Abbott and Regehr, 2004). Our new insight concerning the spatial Ca2+ selectivity of different forms of CaV2.1 regulation suggests a strategy whereby variable blends of CDF and CDI could be produced at different types of synapses. At one extreme, the calyx of Held harbors the only synapse wherein native CaV2.1 CDF and CDI have been firmly established (Borst and Sakmann, 1998; Cuttle et al., 1998; Xu and Wu, 2005). This synapse is atypically large, with more numerous presynaptic channels and looser coupling between channels and release sites than typical for the majority of synapses (Borst and Sakmann, 1999). In this setting, one could imagine the strong presence of both local and global Ca2+ signals, permitting both CDF and CDI to be manifest. By contrast, for more typical synapses with fewer channels per release site and tighter channel-site coupling (Zucker and Regehr, 2002), only local Ca2+ signals would be strongly preserved, whereas global signals might be substantially diminished. The prediction here would be for strong CDF with weakened CDI, thereby favoring a facilitative synaptic profile that biases toward a high-pass or differentiating manner of synaptic transmission (Tsodyks and Markram, 1997).

Generalization of the Enhanced Opening Mechanism of CDF

Thus far, the enhanced opening mechanism accords well with all of the qualitative distinctions of this mechanism (Fig. 3). To explore the quantitative sufficiency of this gating paradigm, we here explicitly undertake kinetic simulations of the major single-channel experimental results. First, we consider averaged experimental results for $P_o$, $P_{oo}$, and $FL$ for unitary Ba2+ currents through EFa channels (Fig. 8 A, gray curves); these should furnish good constraints on the normal gating mode. Since experimental profiles are independent of prepulse depolarization, we have selected data from the prepulse context alone, for simplicity. The solid black curves (Fig. 8 A) show the results of simulations with the parameters displayed on the far left. The parameters shown in black are the major parameters representing this pattern of gating, being strongly constrained by both $P_o$ and $P_{oo}$ data. Gray parameters for deep closed states (on the left end of the normal gating mode) are included to fit nuances of the $FL$ profile, and the gray parameter on the extreme right represents very slow VDI, the inclusion of which subtly improved $P_o$ and $P_{oo}$ fits at large $t$. Second, it is impressive that these same normal gating mode parameters (from Fig. 8 A) produce a very reasonable simulation of the averaged data for unitary Ca2+ currents through EFb channels (Fig. 8 B). As for the first case, only data from the prepulse context are shown, since data are prepulse independent. The notion of entrapment within the same gating mode of EFb channels (using Ca2+), and of EFa channels (using Ba2+), finds strong support from this sufficiency of the same normal mode parameters to simulate both experimental profiles. Third, the experimental data for unitary Ca2+ currents through EFb channels, as present after prepulse depolarization, should provide suitable constraints for the facilitated gating mode viewed in isolation (Fig. 8 C, gray traces). Parameters shown at the left support very reasonable fits to the data (black curves). Interestingly, the key parameter changes within the facilitated mode are mainly restricted to acceleration of the final C→O transition and slowing of the initial O→C transition. This suggests that facilitation mainly involves alterations to both opening and closing transitions very close to the ultimate open conformation. Finally, without change to the previously established parameters for normal and facilitated gating modes, we simulate the experimental profile for unitary Ca2+ currents through EFb channels (Fig. 8 D). The only adjustable parameters concern transitions from normal to facilitated modes of gating. The reasonable agreement of simulations and data strongly supports the quantitative sufficiency of the enhanced opening mechanism.

Regarding a broader mechanistic perspective, we mention that the enhanced opening mechanism for CaV2.1 CDF (Fig. 8) is strikingly reminiscent of the modal switching mechanism that explains CDI of CaV1.2 channels (Imredy and Yue, 1994). The only difference is that Ca2+ drives transition from lower to higher $P_o$ modes in the CDF mechanism, whereas Ca2+ does the inverse for the CDI scheme. Kinetic similarities extend to the manner of gating differences between modes, which in both cases are localized to transitions immediately surrounding the open conformation. Structural similarities include the triggering of both forms of modulation by the C-terminal lobe of CaM (Peterson et al., 1999; DeMaria et al., 2001; Chaudhuri et al., 2005); and the sensitivity of both forms of regulation to similar structural manipulations within the IQ domain (Zuhlke et al., 1999, 2000; DeMaria et al., 2001) and EF-hand locus (de Leon et al., 1995; Zuhlke and Reuter, 1998; Peterson et al., 2000; Chaudhuri et al., 2001, 2004). Given these correlations, it is tempting to speculate that CaV2.1 CDF and CaV1.2 CDI represent structurally analogous mechanisms wherein the polarity of mode switching by Ca2+/CaM has been inverted.

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