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Contributions of SERCA pump and ryanodine-sensitive stores to presynaptic residual Ca^{2+}

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Abstract

The presynaptic Ca^{2+} signal, which triggers vesicle release, disperses to a broadly distributed residual $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_{\text{res}}$) that plays an important role in synaptic plasticity. We have previously reported a slowing in the decay timecourse of $[\text{Ca}^{2+}]_{\text{res}}$ during the second of paired pulses. In this study, we investigated the contributions of organelle and plasma membrane Ca^{2+} flux pathways to the reduction of effectiveness of $[\text{Ca}^{2+}]_{\text{res}}$ clearance during short-term plasticity in Schaffer collateral terminals in the CA1 field of the hippocampus. We show that the slowed decay timecourse is mainly the result of a transport-dependent Ca^{2+} clearance process; that presynaptic caffeine-sensitive Ca^{2+} stores are not functionally loaded in the unstimulated terminal, but that these stores can effectively take up Ca^{2+} even during high frequency trains of stimuli; and that a rate limiting step of sarco-endoplasmic reticulum Ca^{2+} -ATPase (SERCA) kinetics following the first pulse is responsible for a large portion of the observed slowing of $[\text{Ca}^{2+}]_{\text{res}}$ clearance during the second pulse. We were able to accurately fit our $[\text{Ca}^{2+}]_{\text{res}}$ data with a kinetic model based on these observations and this model predicted a reduction in availability of unbound SERCA during paired pulses, but no saturation of Ca^{2+} buffer in the endoplasmic reticulum.

Keywords

Hippocampus; Schaffer collateral; Kinetics; Calcium clearance; Endoplasmic reticulum

1. Introduction

Following a presynaptic action potential, there is a rapid rise of $[\text{Ca}^{2+}]$ in the immediate vicinity of Ca^{2+} channels that triggers release of vesicles docked within this microdomain [1]. This presynaptic Ca^{2+} signal ($[\text{Ca}^{2+}]_{\text{pre}}$) then disperses by diffusion and buffering to produce a residual $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_{\text{res}}$) that decays over the course of tens to hundreds of milliseconds. Presynaptic $[\text{Ca}^{2+}]_{\text{res}}$, although at a lower concentration than that necessary for vesicular release, plays a modulatory role that is important in synaptic plasticity [2,3] and has been implicated as a basis for working memory storage [4]. Thus, the duration of the $[\text{Ca}^{2+}]_{\text{res}}$ signal is a crucial determinant of the dynamic properties of synaptic transmission.

In a previous study [5], we reported that the decay timecourse of $[\text{Ca}^{2+}]_{\text{res}}$ is significantly slowed during the second of paired pulses under conditions where short-term synaptic plasticity is observed. In this study, we have investigated those mechanisms that could alter the decay timecourse of $[\text{Ca}^{2+}]_{\text{res}}$ and hence influence the efficacy of synaptic plasticity. The

predominant pathways that determine this timecourse are: (1) entry via voltage-gated Ca^{2+} channels (VGCCs); (2) passive diffusion or buffering; (3) Ca^{2+} -dependent Ca^{2+} release (CICR) through ryanodine receptors in the endoplasmic reticulum (ER); (4) uptake into the ER by the sarco-endoplasmic reticulum Ca^{2+} -ATPase (SERCA); (5) extrusion across the plasma membrane by either the plasma membrane Na/Ca exchanger (NCX) or the plasma membrane Ca^{2+} -ATPase (PMCA); and (6) uptake into mitochondria by the mitochondrial Ca^{2+} uniporter (MCU) [6,7]. Contributions to the timecourse of $[\text{Ca}^{2+}]_{\text{res}}$ by presynaptic Ca^{2+} buffers and by uptake and release from the ER have been an important focus in previous studies of the involvement of $[\text{Ca}^{2+}]_{\text{res}}$ in short-term plasticity.

Ca^{2+} buffering by endogenous and exogenously introduced buffers is a ubiquitous passive influence on the dispersion of the Ca^{2+} microdomain and on the timecourse of $[\text{Ca}^{2+}]_{\text{res}}$ [8]. In addition, saturation of these buffers can make an important contribution to short-term facilitation in some presynaptic terminals, although its contribution appears to be minimal in the Schaffer collateral terminals in the CA1 subfield of the hippocampus [5,9]. Intracellular Ca^{2+} stores play a crucial role in many facets of neuronal function including synaptic plasticity [10]. Uptake of Ca^{2+} into the presynaptic ER and CICR from these Ca^{2+} stores provides a potentially important modulatory mechanism for neurotransmitter release, although the contribution of presynaptic Ca^{2+} stores to synaptic plasticity depends on the stimulus conditions and the specific synaptic terminal involved. For instance, there is a clear indication of Ca^{2+} stores involvement in long-term synaptic plasticity in the hippocampus including long-term potentiation (LTP) [11] and long-term depression (LTD) [12] and this is consistent with the finding that Ca^{2+} stores make a significant contribution to $[\text{Ca}^{2+}]_{\text{res}}$ following trains of presynaptic action potentials [13]. The degree of involvement of presynaptic Ca^{2+} stores in short-term plasticity appears to be dependent on the specific synapse type. Thus, mossy fiber terminals in the CA3 hippocampal subfield exhibit CICR-dependent short-term facilitation [14], while other synapses of these fibers do not exhibit the same CICR-dependent mechanism [15]. Additionally, CICR has been found to have no involvement in short-term plasticity in Schaffer collateral terminals on CA1 pyramidal neurons in young rats [16], although another study of these same synapses found CICR involvement in short-term plasticity of functional, but not silent synapses [17].

In this study, we have investigated the contributions of VGCCs, CICR, SERCA, NCX, PMCA, and MCU to the timecourse of $[\text{Ca}^{2+}]_{\text{res}}$. We have focused primarily on the slow component of $[\text{Ca}^{2+}]_{\text{res}}$ decay, since this component reflects $[\text{Ca}^{2+}]_{\text{res}}$ clearance processes, which occur on the time scale of short-term synaptic plasticity. We show that there is a slowing of the decay timecourse of $[\text{Ca}^{2+}]_{\text{res}}$ during the second of paired pulses that results from the decrease of a transport-dependent $[\text{Ca}^{2+}]_{\text{pre}}$ clearance process. Presynaptic caffeine-sensitive Ca^{2+} stores do not contain measurable Ca^{2+} in the unstimulated presynaptic terminal, although these stores can take up $[\text{Ca}^{2+}]_{\text{pre}}$ during high frequency trains of stimuli. We find a minimal contribution of VGCCs, NCX, PMCA, or MCU in the observed difference of $[\text{Ca}^{2+}]_{\text{res}}$ decay timecourse between the first and second of paired pulses. However, a limitation of the rate of the SERCA pump following the first presynaptic stimulus is responsible for a large portion of the observed slowing of $[\text{Ca}^{2+}]_{\text{res}}$ decay. Our kinetic modeling studies are consistent with a major role for SERCA and a significant capacity for ER Ca^{2+} storage during $[\text{Ca}^{2+}]_{\text{pre}}$ clearance in Schaffer collateral terminals. Furthermore, SERCA clearance of $[\text{Ca}^{2+}]_{\text{res}}$ appears to be an important contributory factor in the genesis to short-term plasticity.

2. Methods

2.1. Slice preparation and field potential recordings

Experiments were performed in coronal hippocampal slices from approximately 50 day-old Sprague-Dawley rats as previously described [5]. Briefly, animals were deeply anesthetized

with an i.p. injection of 250 mg/kg ketamine, brains were rapidly removed, and slices were cut at 300 μ m with a vibroslicer (Pelco 101, St. Louis, MO) in an ice bath with a cutting solution containing (in mM): 220 sucrose, 3 KCl, 1.2 NaH₂PO₄, 26 NaHCO₃, 12 MgSO₄, 0.2 CaCl₂, 10 glucose, and 0.01 mg/ml ketamine equilibrated with 95% O₂/5% CO₂. Slices were then transferred to artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 5 KCl, 1.25 NaH₂PO₄, 1.3 MgSO₄, 26 NaHCO₃, 2.5 CaCl₂, and 10 glucose equilibrated with 95% O₂/5% CO₂ at 30 °C for 1 hour and then maintained at room temperature until transfer to the recording chamber (Warner Instruments, Hamden, CT), which was maintained at 32 °C, and continuously perfused at 2 ml/min (bath exchange τ = 70 s) with ACSF saturated with 95% O₂/5% CO₂. In ACSF where Ca²⁺ was replaced with Ba²⁺, MgCl₂ was substituted for MgSO₄. We used standard electrophysiological techniques that are well established in our laboratory for slice field potential recordings in the *stratum pyramidale* of the hippocampus [5] using a Multiclamp 700B amplifier (Axon Instruments, Union City, CA), digitized at 500 KHz and filtered at 2 KHz with a Digidata 1322A interface using pCLAMP 10 software (Axon Instruments). Presynaptic constant current pulses (150 μ s duration) were applied to Schaffer collateral fibers with an Iso-Flex constant current stimulator (A.M.P.I Instruments, Jerusalem, Israel) through a concentric bipolar electrode (FHC, Bowdoinham, ME) at a current adjusted to produce ~50% of the maximum fEPSP population spike amplitude. For simplicity, we have used the abbreviation R1 for the first response to a pair of stimuli and R2 for the second response of the pair. All experiments were approved by the Institutional Animal Care and Use Committee at the University of New Mexico Health Sciences Center and conformed with NIH guidelines.

2.2. Presynaptic Ca²⁺ imaging

Presynaptic fibers were filled with the Ca²⁺ fluorophore, Mg Green AM, Fura-2 AM, or FuraFF AM using an established technique that allowed simultaneous measurements of presynaptic [Ca²⁺]_i and postsynaptic fEPSP [5,18–21]. In a small number of instances, either the presynaptic or postsynaptic recording was not successful, but the data from the successful component were still included. To minimize the effect exogenous buffers, we used Mg-Green or FuraFF, with Ca²⁺ binding K_D s between 5 and 6 μ M for experiments in which we measured the timecourse of $\Delta F/F_0$ decay [19,22]; however, in some experiments where the rapid decay timecourse was not being assessed, we improved the sensitivity of the fluorescence measurements by using the higher affinity fluorophore Fura-2. Briefly, an ejection electrode (5–10 μ m tip diameter) containing the fluorophore (0.9mM Mg-Green AM, Fura-2 AM, or Fura-FF AM (Invitrogen, Eugene, OR); 10% v/v DMSO; 1% v/v pluronic acid in ACSF) was inserted into the fiber pathway between the stimulating electrode and the presynaptic terminal field to be observed. While monitoring the emission image during excitation at 490nm for Mg-Green or 350nm for Fura-2 or Fura-FF, an air pressure pulse was applied with a syringe to the ejection electrode until a small bright spot (estimated to be $\cong$ 1 μ l) was observed in the fiber pathway. The slice was then maintained with a 2 ml/min flow of oxygenated ACSF at 32°C for ~1 h to allow intracellular diffusion of the dye to the presynaptic imaging site ~500 μ m away from the ejection site. For single wavelength studies with Mg-Green or Fura-FF, the excitation light was then reduced with a diaphragm in the epi-illumination path to a 100–200 μ m diameter spot and the emitted light was measured from this Schaffer collateral terminal field with a photomultiplier tube (PMT). For ratiometric studies with Fura-2, randomly chosen regions of interest were analyzed within the Schaffer collateral terminal field in stratum radiatum. A single stimulus or pairs of stimuli were delivered orthodromically at 0.067 Hz by a Master 8 pulse generator (A.M.P.I Instruments) under control of the imaging system (T.I.L.L. Photonics, Pleasanton, CA). Fluorescent responses are reported as the ratio of the change in fluorescence to the pre-stimulus fluorescence ($\Delta F/F_0$) for Mg-Green or Fura-FF studies or as the change in 350/380 nm excitation ratios ($\Delta R/R_0$) for Fura-2 studies. The fluorescent signals were corrected for bleaching by subtracting a linear baseline and were inverted so that increasing [Ca²⁺]_{pre} produced an upward deflection. Time integrals of the $\Delta F/F_0$ ($\int \Delta F/F_0$) were

calculated to provide a measure of total presynaptic $[Ca^{2+}]_i$ during the period of short-term facilitation [5].

We used two tests to demonstrate that the measured $\Delta F/F_0$ signal was consistent with a $[Ca^{2+}]$ response predominately from the presynaptic Schaffer collateral axons and axon terminals [5,19]. First, in the presence of 10 μ M CNQX, 25 μ M D-AP5, and 20 μ M bicuculline, the fEPSP was blocked, while the presynaptic fiber volley and $\Delta F/F_0$ signal were left unchanged, thus the $\Delta F/F_0$ signal was not a postsynaptic response. Second, subsequent addition of 600 nM TTX blocked both the presynaptic fiber volley and the $\Delta F/F_0$ signal arguing against direct stimulation of inadvertently filled postsynaptic dendrites. Because of the noise inherent in measurements with the low affinity indicators, it was necessary to average five fluorescence responses and to filter the PMT signal at 1 kHz. Some signals were further post filtered with a Gaussian filter with a 3 dB cut off at 300 Hz. Paired-pulse and single-pulse responses were collected under each condition and average single-pulse responses were subtracted from average paired-pulse responses to generate the Ca^{2+} residual-subtracted R2 response ($\Delta R2 [Ca^{2+}]_{res}$) as described in the Results.

In control experiments with sister slices in ACSF, we compared Fura-FF and Mg-Green responses to single pulses. While the slow component of the decay was similar, there was a reduction of the fast component in the first 25ms in the Fura-FF data perhaps due to a difference in kinetics of the fluorophores; we did not further pursue the mechanism of this difference. We were unable to accurately fit Fura-FF $\Delta F/F_0$ decay signals with a double exponential as we did with Mg-Green signals and the decay τ for the single exponential fit to the Fura-FF decay was about 27% faster than the τ_s for the double exponential fit to the Mg-Green traces.

2.3. Drugs

Concentrated stock solutions of drugs were mixed at a minimum 1000 $\times$ concentration in appropriate vehicles, stored frozen in aliquots, and diluted on the day of the experiment to the appropriate concentration in ACSF. Thapsigargin was obtained from Tocris (Ellisville, MO), while nimodipine, 5(6) carboxyeosin diacetate, cyclopiazonic acid, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), oligomycin, and benzamil were from Sigma-Aldrich (St. Louis, MO). Absorption spectra were obtained for all drugs at the appropriate concentration in ACSF. With the exception discussed below for carboxyeosin, none of the drugs showed significant absorbance at either the excitation or emission wavelength of the relevant indicators. Drugs were applied through a bath perfusion system and a minimum of 2.5 bath exchanges was allowed after changing solutions before recording. Control experiments at equivalent DMSO concentrations showed no effect of vehicle alone.

2.4. Curve fitting and statistics

Data for the decay from the peak of $\Delta F/F_0$ with Mg-Green were fit using a least squares regression fit to:

$$F=A \left[B e^{-t/\tau_f} + (1-B) e^{-t/\tau_s} \right] \quad (1)$$

where A is the initial value of $\Delta F/F_0$, τ_f and τ_s are respectively the fast and slow time constants of decay, and B is the fraction of the curve fit with τ_f .

Data for the decay from the peak of $\Delta F/F_0$ with Fura-FF were fit using a least squares regression fit to:

$$F = Ce^{-t/\tau} \quad (2)$$

where C is the initial value of $\Delta F/F_0$ and τ is the time constant of decay.

Data for complete $\Delta F/F_0$ traces in the nimodipine experiments were fit using a least squares regression fit to:

$$F = D \left(1 - e^{-t/\tau_{\text{rise}}} \right)^n \left[e^{-t/\tau_{f1}} + e^{-t/\tau_{s1}} \right] + E \quad (3)$$

where D is the initial value of $\Delta F/F_0$, τ_{rise} is the rising time constant, τ_{f1} and τ_{s1} are respectively the fast and slow time constants of decay, E is the $\Delta F/F_0$ offset, and n determines the steepness of the rise.

Data are presented as mean $\pm$ S.E.M. Students t -tests and ANOVAs were calculated with SPSS (SPSS Inc., Chicago, IL) or Pro-Stat (Poly Software, Pearl River, NY) statistics packages. Statistical significance was determined at $p < 0.05$ and indicated as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$.

3. Results

The mechanisms responsible for the regulation of presynaptic $[\text{Ca}^{2+}]_{\text{res}}$, have been well characterized in certain specialized synapses, but they are less well understood in the more representative small presynaptic terminals of the CNS [23]. We therefore measured $[\text{Ca}^{2+}]_{\text{res}}$ during paired stimuli at a 50ms interpulse interval in Schaffer collateral terminals in stratum radiatum of the CA1 subfield of the hippocampus. This interpulse interval was selected because it elicits a form of short-term plasticity that is manifest as an enhancement of neurotransmission, which has been associated with learning and memory. As expected, the peak $[\text{Ca}^{2+}]_{\text{res}}$ following the second pulse (R2) was larger than that following the first pulse (R1) because of persisting $[\text{Ca}^{2+}]_{\text{res}}$ from R1 (Fig. 1A1). To obtain an independent measure of $[\text{Ca}^{2+}]_{\text{res}}$ during the second pulse, we subtracted the $[\text{Ca}^{2+}]_{\text{res}}$ produced by a single pulse (P1) from R2 $[\text{Ca}^{2+}]_{\text{res}}$ to yield $\Delta R2$ $[\text{Ca}^{2+}]_{\text{res}}$. (Note, the distinction between P1 and R1 $[\text{Ca}^{2+}]_{\text{res}}$ is simply that P1 $[\text{Ca}^{2+}]_{\text{res}}$ decays completely while R1 $[\text{Ca}^{2+}]_{\text{res}}$ decay is interrupted by the onset of R2.) As we have shown previously, there is a significant increase in the integral of $\Delta R2$ $[\text{Ca}^{2+}]_{\text{res}}$ over that of P1 $[\text{Ca}^{2+}]_{\text{res}}$ (Fig. 1A2 and B). When measured with the low affinity indicator Mg-Green, the $[\text{Ca}^{2+}]_{\text{res}}$ decay timecourse for P1, and $\Delta R2$ are accurately fit with double exponentials, which yield fast and slow time constants that differ by approximately an order of magnitude [5,7]. The larger integral of $\Delta R2$ $[\text{Ca}^{2+}]_{\text{res}}$ results largely from an increase in the slow time constant (Fig. 1C), although there is also a smaller and less consistent increase in the fast time constant for this decay. There is, however, very little change in the magnitude of the relative contributions of the fast and slow components to the total $[\text{Ca}^{2+}]_{\text{res}}$ decay [5].

3.1. Contribution of transport-dependent processes to $[\text{Ca}^{2+}]_{\text{res}}$

Barium ions readily permeate Ca^{2+} channels, but they are ineffectively eliminated from the cytoplasm of the presynaptic terminal by Ca^{2+} transporters (e.g. [24]). Thus a comparison of the time-course of the fluorescent indicator signals in Ba^{2+} vs. Ca^{2+} can be used to assess the transport-dependent components of $[\text{Ca}^{2+}]_{\text{res}}$ clearance. We measured simultaneously presynaptic $\Delta F/F_0$ and the postsynaptic fEPSP and, as has been well established at many synapses (e.g. [25]), observed that replacing Ca^{2+} with Ba^{2+} in the ACSF quickly and reversibly eliminated the fEPSP, although the transient $\Delta F/F_0$ response persisted (Fig. 2A). The magnitude, but not the timecourse, of this presynaptic $\Delta F/F_0$ signal is affected by the relative

permeabilities of Ca channels to Ba^{2+} vs. Ca^{2+} and by the differential intensities of fluorescence emission of Mg-Green to the two divalent ions. The representative traces in Fig. 2B show the typical increase in the time constant of the slow component (τ_s) of the $\Delta F/F_0$ decay and the almost complete elimination of the fast component (τ_f) of this signal. This reversible increase in τ_s and reduction in the percent of τ_f (B in Eq. (1)) is summarized in Fig. 2C.

Although it was not feasible to directly compare absolute values of the $\int \Delta F/F_0$ in Ca^{2+} and Ba^{2+} when each $\int \Delta F/F_0$ record was normalized to its respective value for P1, there was a significant reduction in the difference between P1 $\int \Delta F/F_0$ and $\Delta R2 \int \Delta F/F_0$ when comparing Ca^{2+} ACSF to Ba^{2+} ACSF (Fig. 2D) suggesting that a transport-dependent process makes an important contribution to the difference in $\int \Delta F/F_0$.

To further test the role of processes other than diffusion or Ca^{2+} buffering as the mechanism for the difference between P1 $\int \Delta F/F_0$ and $\Delta R2 \int \Delta F/F_0$, we measured these integrals at temperatures between 23 and 32°C. From the slopes of the Arrhenius plots of the logarithm of the ratio of $\Delta R2 \int \Delta F/F_0$ to P1 $\int \Delta F/F_0$ vs. the reciprocal of temperature (Fig. 2E), we determined an average $Q_{10} = 2.94 \pm 0.64$ for the underlying process. This is significantly above the minimum expected $Q_{10} = 1.3$ for an active process. In contrast, we used the same technique to measure the temperature dependence of the P1 $\int \Delta F/F_0$ decay in Ba^{2+} ACSF and determined a $Q_{10} = 1.08 \pm 0.32$ ($n = 5$), which is not significantly different from the expected Q_{10} for a passive temperature-independent process (data not shown).

Together these data indicate that a transport-dependent process rather than changes in diffusion or buffering is more effective during R1 than during R2 and this causes R1 $\Delta F/F_0$ to decay more rapidly than $\Delta R2 \Delta F/F_0$. The following experiments were designed to investigate the nature of this process.

3.2. Requirement for preloading of the ER

In many cell types, thapsigargin, an irreversible SERCA pump inhibitor, causes a rapid rise in $[\text{Ca}^{2+}]_i$ due to an uncompensated leak of Ca^{2+} from the ER lumen into the cytoplasm. Even using the more sensitive fluorophore Fura-2, we were unable to detect a significant increase in presynaptic $\Delta R/R_0$ in the presence of 3 μM thapsigargin (Fig. 3A). However, subsequent depolarization with 40 mM K^+ to activate VGCCs led to the expected dramatic increase in $\Delta R/R_0$. We next used 20 mM caffeine, which increases the Ca^{2+} sensitivity of ryanodine receptors and causes release of Ca^{2+} from ER stores, and again we did not find a significant increase in $\Delta R/R_0$. However, when we preceded 10 mM caffeine application by preloading the ER stores with a brief depolarization induced with 40 mM K^+ , we did observe a significant caffeine-dependent increase in $\Delta R/R_0$ (Fig. 3B). Importantly, there was no increase in $\Delta R/R_0$ following the same preloading protocol when no caffeine was applied. Finally, we investigated the effect of repetitive presynaptic stimuli on the loading of ER stores. Two 100 Hz trains of 20 pulses separated by 15 s led to a maintained increase in $\Delta R/R_0$, but there was no further increase after application of 10 mM caffeine. Two higher frequency, 500 Hz trains of 20 pulses, however produced a maintained increase in $\Delta R/R_0$ that was further increased by the subsequent addition of 10 mM caffeine (Fig. 3C). From these experiments, we conclude that neither uncompensated leak following block of SERCA nor ryanodine receptor activation is capable of releasing measurable Ca^{2+} from ER stores in the basal, unstimulated state of these presynaptic terminals in the adult hippocampus. However, depolarization or high frequency stimulation is capable of filling these stores sufficiently for caffeine to cause a measurable Ca^{2+} release. Our preloading experiments further suggest that the presynaptic ER is capable of sequestering a considerably greater cytoplasmic Ca^{2+} load than is produced by pairs of pulses.

3.3. Contribution of sarco-endoplasmic reticulum Ca^{2+} -ATPase (SERCA) to $[\text{Ca}^{2+}]_{\text{res}}$

Since caffeine-sensitive Ca^{2+} stores could be filled following presynaptic Ca^{2+} influx (Fig. 3B and C), it was apparent that SERCA pumps were functional in the presynaptic terminals of our preparation. We thus reasoned that the increase in $\Delta\text{R2 } \int \Delta F/F_0$ could result from a limitation imposed by the rate-determining step of the SERCA pump in clearing the increased $[\text{Ca}^{2+}]_{\text{pre}}$ introduced by the second of paired Ca^{2+} influxes. As can be seen in the example in Fig. 4A, thapsigargin caused an increase in the P1 $\int \Delta F/F_0$ so that it approximately equaled $\Delta\text{R2 } \int \Delta F/F_0$. The summary data in Fig. 4B indicate that, when normalized to the ACSF P1 $\int \Delta F/F_0$, there is not only the expected significant increase in $\Delta\text{R2 } \int \Delta F/F_0$ in both ACSF and thapsigargin, but there is also an increase in P1 $\int \Delta F/F_0$ in thapsigargin. As a consequence, there was no significant difference between P1 $\int \Delta F/F_0$ and $\Delta\text{R2 } \int \Delta F/F_0$. We also carried out confirmatory experiments with cyclopiazonic acid, which is another specific inhibitor of SERCA pumps (see Fig. 5E). Again, we found that P1 $\int \Delta F/F_0$ in cyclopiazonic acid was increased so that it approximately equaled $\Delta\text{R2 } \int \Delta F/F_0$.

As shown in Fig. 4C, when the decay phase of $\Delta F/F_0$ following a single pulse was fit with a double exponential (Eq. (1)), there was a significant increase of τ_s in thapsigargin. There was also a small increase in the initial amplitude of $\Delta F/F_0$ that was quite pronounced in some slices while changes in τ_f and in $\% \tau_f$ (B in equation 1) were neither consistent nor significant. In control experiments, in which the vehicle DMSO was applied alone for an equivalent time, there was no significant change in either P1 $\int \Delta F/F_0$ ($p = 0.65$) or $\Delta\text{R2 } \int \Delta F/F_0$ ($p = 0.63$) (data not shown, $n = 7$). Our interpretation of these results is that SERCA pumps play an important role in $[\text{Ca}^{2+}]_{\text{res}}$ clearance after R1 and that they become rate limited thereby increasing $\Delta\text{R2 } \int \Delta F/F_0$. As a result, thapsigargin increases P1 $\int \Delta F/F_0$ and eliminates its difference from $\Delta\text{R2 } \int \Delta F/F_0$.

3.4. Contribution of plasma membrane Ca^{2+} flux to $[\text{Ca}^{2+}]_{\text{res}}$

Presynaptic P/Q-, N-, and R-type VGCCs play an essential role in transmitter release [26] and in the generation of $[\text{Ca}^{2+}]_{\text{res}}$. Although L-type VGCCs are not involved in fast synaptic transmission, there is evidence that they may contribute to changes in synaptic efficacy [27, 28] and an indication of coupling between L-type VGCCs and RyRs [29]. Therefore we considered whether activation of these channels could play a role in the increase of $\Delta\text{R2 } \int \Delta F/F_0$ by measuring the $\int \Delta F/F_0$ in the presence of the selective L-type VGCC blocker nimodipine (10 μM). This treatment did not cause a significant change in the shape of the rising portion of the $\int \Delta F/F_0$ response when we fit the entire $\Delta F/F_0$ trace with equation 3 (data not shown). When we fit only the falling portion of $\Delta F/F_0$ with equation 1, there was a significant 22% decrease in τ_f , although none of the other parameters—in particular τ_s —was significantly changed. Furthermore, the increase in $\Delta\text{R2 } \int \Delta F/F_0$ over P1 $\int \Delta F/F_0$ persisted following block of L-type VGCCs (Fig. 5A1 and C). We interpret this to mean that there is not an important contribution of L-type VGCCs to the increase of $\Delta\text{R2 } \int \Delta F/F_0$.

We initially attempted to reduce NCX by replacing external Na^+ with Li^+ , but found that in some slices this led to the elimination of $\Delta F/F_0$ responses, probably due to secondary effects of Na^+ depletion causing spreading depression in the slice [30]. We thus assessed the role of NCX in the increase of $\Delta\text{R2 } \int \Delta F/F_0$ with the selective blocker of NCX, 100 μM benzamil. A complication of using benzamil, however, is that it has been reported to also block L-type VGCCs [31] so we carried out these experiments in the presence of the L-type VGCC blocker, nimodipine. Block of NCX neither significantly altered P1 $\int \Delta F/F_0$ nor did it eliminate the increase of $\Delta\text{R2 } \int \Delta F/F_0$ over P1 $\int \Delta F/F_0$ (Fig. 5A2 and D). We conclude that NCX is not an important contributor to $[\text{Ca}^{2+}]_{\text{res}}$ clearance during pairs of pulses at 50ms interpulse interval and that its partial saturation or reduction in effectiveness is not a major factor in the increase in $\Delta\text{R2 } \int \Delta F/F_0$.

We used 40 μ M 5(6)-carboxyeosin diacetate to block the PMCA pumps in order to assess the contribution of this transmembrane pathway to $[Ca^{2+}]_{res}$ clearance. Carboxyeosin has a fluorescence emission (542 nm), which is similar to that for Mg-Green, and this produced a background emission that saturated the PMT. Thus, we chose to conduct these experiments with Fura-FF, which has a shorter emission wavelength and a K_D for Ca^{2+} (5.5 μ M) that is similar to that of Mg-Green K_D (6 μ M). As discussed in the Methods, the fast component of presynaptic $\Delta F/F_0$ signal in Fura-FF was reduced over that seen in sister slices with Mg-Green and thus the Fura-FF responses were best fit with a single exponential (equation 2). In addition, there is evidence that carboxyeosin can inhibit SERCA pumps [32] so 10 μ M cyclopiazonic acid was used throughout these experiments to block SERCA pumps. Fig. 5B1 and 5E show expected control conditions in which the difference P1 $\int \Delta F/F_0$ and $\Delta R2 \int \Delta F/F_0$ was eliminated as a result of block of SERCA pumps by cyclopiazonic acid. Addition of 40 μ M carboxyeosin did not produce an additional change in either P1 $\int \Delta F/F_0$ or in $\Delta R2 \int \Delta F/F_0$ (Fig. 5B2 and E).

3.5. Contribution of mitochondrial Ca^{2+} uniporter (MCU) to $[Ca^{2+}]_{res}$

In order to assess the contribution of the MCU to $[Ca^{2+}]_{res}$ clearance, we were constrained to using the indirect approach of abolishing the mitochondrial membrane potential ($\Delta\psi_m$) with the protonophore, FCCP (1 μ M), [33]. In initial experiments, FCCP alone led to a significant decrease in both the P1 $\int \Delta F/F_0$ and the $\Delta R2 \int \Delta F/F_0$ and it also eliminated the postsynaptic fEPSP response. We decided therefore to include 2.5 μ g/ml oligomycin, an inhibitor of the F_0 portion of mitochondrial H^+ -ATP-synthase, to prevent rapid ATP consumption following dissipation of the H^+ gradient by FCCP [34]. Surprisingly, oligomycin alone eliminated the expected increase of $\Delta R2 \int \Delta F/F_0$ over P1 $\int \Delta F/F_0$; however, we observed further that this effect of oligomycin resulted from a significantly decreased contribution of the slow component to the overall $\Delta F/F_0$ decay (Fig. 5F2) with no significant effect on τ_f (Fig. 5F3). Importantly, when we determined τ_s in oligomycin or in oligomycin plus 1 μ M FCCP, we found the usual increase in τ_s of the decay of $\Delta R2 \int \Delta F/F_0$ over that of R1 $\int \Delta F/F_0$ (Fig. 5F1). As we will consider further in the Discussion, we interpret these results to reflect largely an action of oligomycin on mitochondrial ATP production rather than a major contribution of the MCU to $[Ca^{2+}]_{res}$ clearance.

3.6. Relationship of $[Ca^{2+}]_{res}$ clearance and short-term facilitation

To further evaluate the role of $[Ca^{2+}]_{res}$ clearance in short-term facilitation of synaptic transmission, postsynaptic responses were measured simultaneously with the presynaptic $\Delta F/F_0$ signal. We anticipated that changes in the timecourse of $[Ca^{2+}]_{res}$ clearance could affect the magnitude of short-term facilitation. In particular, manipulations that cause an increase in τ_s should result in greater paired-pulse facilitation (PPF) [5]. Thapsigargin or cyclopiazonic acid with carboxyeosine, either of which increased P1 $\int \Delta F/F_0$ (Fig. 4B and Fig. 5E), caused in a significant increase in PPF (Fig. 6). In contrast, nimodipine plus benzamil or oligomycin plus FCCP, neither of which increased P1 $\int \Delta F/F_0$ (Fig. 5B and Fig. 5D), did not significantly affect PPF (Fig. 6B).

3.7. Modeling of $[Ca^{2+}]_{res}$ clearance

We reported previously [5] that the increase of $\Delta R2 \int \Delta F/F_0$, is primarily due to an increase in τ_s of $[Ca^{2+}]_{res}$ clearance (Fig. 1). Our observations that this clearance timecourse is best fit with a double exponential and that SERCA pumps play a central role in the process were important guidelines for creating a simplified model for $[Ca^{2+}]_{res}$ clearance (see Appendix A).

We approximated passive clearance processes as a single component, which we estimated from our experiments in Ba^{2+} ACSF. We began with a complete kinetic model for $[Ca^{2+}]_{res}$ clearance, but then reduced it to only those kinetic components associated with passive diffusion and ER Ca^{2+} flux (Fig. 7A bold). We assumed that the rate limiting steps of SERCA

transport were associated with Ca^{2+} binding in the cytoplasm and ER lumen and not with the kinetics of ATP-dependent translocation across the ER membrane. We used the Potterswheel program [35] to perform a sensitivity analysis and took special note of those parameters that were most sensitive to producing an accurate fit of the $\Delta F/F_0$ data. The kinetic model was then fit to $\Delta F/F_0$ data from Fig. 1A 1 using numerical integration with MATLAB® software (MathWorks, Natick, Massachusetts) (Fig. 7B). The same initial boundary conditions were fit to 23 additional $\Delta F/F_0$ traces of Ca^{2+} influx following pairs of pulses separated by 50ms that were recorded under control conditions in ACSF. The $\Delta F/F_0$ data were scaled to the estimated Ca^{2+} influx during a single pulse and average parameters were determined that provided timecourse information for binding of cytoplasmic Ca^{2+} to SERCA (Fig. 7C). To further test this model, we fit $[\text{Ca}^{2+}]_{\text{res}}$ data resulting from a short train of five pulses in order to predict the timecourse of ER Ca^{2+} buffering (Fig. 7D). The $\Delta F/F_0$ data were scaled as in Fig. 7C in order to predict binding of Ca^{2+} to SERCA and the concentration of buffered ER Ca^{2+} ($[\text{CaB}]_{\text{ER}}$).

The applicability of the reduced kinetic model to the process of $[\text{Ca}^{2+}]_{\text{res}}$ clearance is demonstrated in four ways. First, the model generates a fit to the $[\text{Ca}^{2+}]_{\text{res}}$ decay data that is comparable to that of a double exponential. For instance, the decay portion of the $\Delta F/F_0$ data for the single pulse of Fig. 1A1 yields an $R^2 = 0.92$ when fit with the model and an $R^2 = 0.93$ when fit with a double exponential. Second, the model predicts the observed increase of $\Delta R2 \int \Delta F/F_0$ over P1 $\int \Delta F/F_0$. For example, the modeled $[\text{Ca}^{2+}]_{\text{res}}$ for P1 and $\Delta R2$ in Fig. 7C gives a ratio of $\Delta R2 \int \Delta F/F_0$ to P1 $\int \Delta F/F_0$ of 1.34, while the average value of this ratio for the data from each of the 23 individual experiments is 1.33. Third, consistent with our data, the model predicts a progressive limitation in the availability of SERCA for binding cytoplasmic free Ca^{2+} ($[\text{Ca}^{2+}]_{\text{cyt}}$) for transport into the ER (Fig. 7C and D). Fourth, also consistent with our data, the model predicts that ER Ca^{2+} buffer is not saturated over short trains of pulses (Fig. 7D).

4. Discussion

In a previous study of the role of Ca^{2+} dynamics in governing short-term facilitation, we showed that the $\Delta R2 \int \Delta F/F_0$ was increased over P1 $\int \Delta F/F_0$ and that this increased presynaptic $[\text{Ca}^{2+}]_{\text{res}}$ signal was due largely to an increase in τ_s for $\Delta F/F_0$ decay [5]. We proposed that this was indicative of changes in the buffering, sequestering, or extrusion of Ca^{2+} in the presynaptic terminals and designed these studies to investigate contributions from these mechanisms. In order to assess the contributions of the processes that regulate $[\text{Ca}^{2+}]_{\text{pre}}$ on the timescale of short-term synaptic plasticity, we used pharmacological agents to independently block individual influx and clearance pathways and then measured the effects on R1 and $\Delta R2 \int \Delta F/F_0$. While some of our results were complicated by significant interactions of certain drugs on these pathways, we were able to clearly delineate a major role for the SERCA pathway into the ER on the timecourse of $[\text{Ca}^{2+}]_{\text{res}}$ clearance. In addition, our $\Delta F/F_0$ data were accurately fit with a model reflecting SERCA kinetics under conditions where there was a reduction during R2 of available SERCA Ca^{2+} binding for the removal of free Ca^{2+} from the cytoplasm. Importantly, inhibition of this pathway has important consequences in short-term plasticity.

When Ba^{2+} was substituted for Ca^{2+} in the ACSF, the portion of $\Delta F/F_0$ decay fit by a fast exponential was approximately halved (Fig. 2C), τ_s was approximately doubled (Fig. 2C), and the enhancement of $\Delta R2 \int \Delta F/F_0$ was eliminated (Fig. 2D). The temperature dependence of the increase in the $\Delta R2 \int \Delta F/F_0$ yielded a Q_{10} , which was significantly above the minimum expected for an active process (Fig. 2E). Thapsigargin did not produce a significant increase in $[\text{Ca}^{2+}]_{\text{pre}}$ (Fig. 3A) and caffeine did not cause an increase in $[\text{Ca}^{2+}]_{\text{pre}}$ unless the presynaptic terminals had been preloaded with either high K^+ or by high frequency stimulation (Fig. 3 B and C). Neither nimodipine (Fig. 5C) nor benzamil (Fig. 5D) significantly altered the

enhancement of $\Delta R2 \int \Delta F/F_0$ and following application of carboxyeosin there was no additional change in the $\int \Delta F/F_0$ over that already produced by cyclopiazonic acid (Fig. 5E). The increase in τ_s during $\Delta R2 \int \Delta F/F_0$ persisted when FCCP was applied in the presence of oligomycin (Fig. 5F1). Thapsigargin increased P1 $\int \Delta F/F_0$, largely due to an increase in τ_s (Fig. 4C) and eliminated the significant difference between P1 $\int \Delta F/F_0$ and $\Delta R2 \int \Delta F/F_0$ (Fig. 4A and B). Interestingly, neither L-type VDCCs nor sequestering of $[Ca^{2+}]_{res}$ by the MCU contributed to τ_s , but they both appeared play a role in some faster process reflected in τ_f , possibly through an effect of $[Ca^{2+}]_{pre}$ or ATP on SERCA kinetics. Uptake of Ca^{2+} into lysosomes by the vacuolar proton pump has been identified as an additional Ca^{2+} clearance pathway in axon terminals [36]; however, we were unable to evaluate the contribution of this pathway in these studies.

4.1. Contribution of transport-dependent Ca^{2+} flux to $[Ca^{2+}]_{res}$

Barium has been used frequently as a substitute for Ca^{2+} as a permeant ion in biophysical studies of I_{Ca} , since it readily permeates most Ca^{2+} channels [37]. Importantly though, Ba^{2+} is cleared one to two orders of magnitude more slowly from synaptic terminals than is Ca^{2+} [38] because it is ineffectively removed from the cytoplasm by transporters including SERCA pumps [39], the NCX [24], the PMCA [40] (but see: [41]), and the MCU [24]. The ability of Ba^{2+} to bind to presynaptic cytoplasmic Ca^{2+} -binding proteins is less clear, although it is ineffective in activating calmodulin-like [42] or troponin-like [43] binding sites. We thus chose to use ACSF in which Ca^{2+} was replaced with Ba^{2+} to help isolate transport-dependent components of $[Ca^{2+}]_{res}$ decay.

Our data show a halving of the portion of $\Delta F/F_0$ that is fit with a fast exponential and a doubling of τ_s in Ba^{2+} (Fig. 2C). The simplest explanation for these observations is that much of the rapid phase and half of the slow phase of $[Ca^{2+}]_{res}$ decay results from transport-dependent Ca^{2+} clearance from the cytoplasm. Likewise, the elimination of the increase of the $\Delta R2 \int \Delta F/F_0$ in the presence of Ba^{2+} (Fig. 2D) is consistent with our hypothesis that this increase results from the reduction of a transport-dependent process of Ca^{2+} sequestering or extrusion during the second of paired pulses. In further support of this hypothesis, the increase of $\Delta R2 \int \Delta F/F_0$ over P1 $\int \Delta F/F_0$ has a temperature dependence that is significantly above the minimum of 1.3 expected for an active process (Fig. 2E).

4.2. Contribution of ER Ca^{2+} stores to $[Ca^{2+}]_{res}$

Presynaptic ER contains functional ryanodine receptors [44] and the ER is found in close proximity to vesicle release sites [45]. Thus ER could potentially be both a contributor to $[Ca^{2+}]_{pre}$ uptake and a source for CICR that could contribute to short-term synaptic plasticity and to the increase in the $\Delta R2 \int \Delta F/F_0$ that we have observed. In Schaffer collateral terminals, while presynaptic CICR has been clearly shown to play a role in stimulus-induced LTD [46, 47] and caffeine-induced LTP [48], the role of CICR in presynaptic short-term facilitation is controversial [49]. For example, ryanodine or caffeine has been shown to decrease PPF and thapsigargin to transiently increase PPF in slices from young rats [17]. In contrast, thapsigargin or ryanodine have been reported to have no effect on PPF or $[Ca^{2+}]_{pre}$ in a similar preparation [16]. In our study, a rather high frequency loading stimulus was required in order to permit measurable Ca^{2+} release in the presence of caffeine while lower frequency preloading paradigms caused a maintained elevation of $[Ca^{2+}]_{pre}$ with no further increase upon the addition of caffeine (Fig. 3B and C). The slow application timecourse inherent in our bath exchange protocol, by allowing time for establishment of a new equilibrium between SERCA uptake from and ryanodine-dependent release into the cytoplasm, may have contributed to our difficulty in obtaining measurable release from loaded ER stores. However, our data clearly indicate that the ER Ca^{2+} stores in Schaffer collateral presynaptic terminals in adult rats only release significant Ca^{2+} if they are first preloaded. Furthermore, it is not surprisingly that under

basal conditions in our experiments, preventing ER Ca^{2+} uptake by blocking the SERCA pump with thapsigargin did not produce a significant elevation of $[\text{Ca}^{2+}]_{\text{pre}}$ (Fig. 3A).

The use of caffeine to cause Ca^{2+} release from ER stores through its action on ryanodine receptors is complicated because of its action on presynaptic adenosine receptors. It has been reported that caffeine can relieve the tonic inhibition produced by extra-cellular adenosine [50] and thereby increase Ca^{2+} influx through VGCCs [48]. However, since we have observed CICR only following preloading of the intracellular stores, it is unlikely that the increase in $[\text{Ca}^{2+}]_{\text{pre}}$, which we observe (Fig. 3B and C), occurs solely by this mechanism. A further complication lies in the effect of millimolar concentrations of caffeine on measurements made with fluorescent indicator dyes. While this effect should be minimized by using the ratiometric dye Fura-2 [51], it may still have led to a reduction in the $[\text{Ca}^{2+}]_{\text{pre}}$ signal both with and without stores preloading. Importantly, our Fura-2 measurements were sufficiently sensitive to determine the increase in $[\text{Ca}^{2+}]_{\text{pre}}$ that follows 2×20 pulses at 100 Hz (Fig. 3C) and the caffeine-dependent release from stores that had previously been loaded by a 30 s depolarization (Fig. 3B).

Further published evidence for the role of CICR in presynaptic terminals comes from the use of thapsigargin, which can deplete ER Ca^{2+} stores through a significant Ca^{2+} leak in the absence of compensatory uptake by SERCA pumps. In slices from young rats at room temperature, thapsigargin decreased PPF in minimal stimulation studies of functional, but not silent, Schaffer collateral terminals in CA1 [17]. In contrast, Carter et al. [16] found that thapsigargin did not affect PPF of evoked EPSCs nor did it affect the $t_{1/2}$ of decay of $[\text{Ca}^{2+}]_{\text{res}}$ for a single pulse. In our studies of Schaffer collateral terminals in adult hippocampal slices, block of SERCA pumps did not produce a measurable increase in $[\text{Ca}^{2+}]_{\text{pre}}$ under basal conditions (Fig. 3A). This is consistent with our finding that these stores are not significantly loaded under those conditions, although this could also indicate that there is minimal passive leak from the ER in these terminals. Moreover, our data reveal that SERCA pumps provide an important contribution to the difference between P1 $\Delta F/F_0$ and $\Delta R2 \Delta F/F_0$ (Fig. 4B) and their block leads to an enhancement of PPF (Fig. 6). This implies that a component of the SERCA removal mechanism becomes less effective following the R1 Ca^{2+} influx. One potential mechanism for this reduced effectiveness is that the cytoplasmic binding site of the SERCA pump becomes partially saturated as a result of the R1 Ca^{2+} influx. Another possibility is suggested by the observation in rat brain microsomes that SERCA activity decreases in response to increasing ER luminal $[\text{Ca}^{2+}]$ [52]. Either of these mechanisms would affect the timecourse of available SERCA during paired or repetitive pulses as predicted by our model (Fig. 7C and D).

4.3. Contribution of plasma membrane flux to $[\text{Ca}^{2+}]_{\text{res}}$

Presynaptic $[\text{Ca}^{2+}]_{\text{res}}$ could be influenced by flux across the plasma membrane through Ca^{2+} permeable channels, exchangers, or pumps and we considered use-dependent changes in each of these pathways as a possible source for the increase in $\Delta R2 \Delta F/F_0$. While L-type VGCCs in Schaffer collateral terminals play a role following high frequency stimulation [53] and specifically in LTP [11,54] and in LTD [55], we found a small effect of blocking L-type Ca^{2+} channels only on the τ_f of $\Delta F/F_0$ decay. This could reflect the block of a persistent Ca^{2+} influx into a compartment that is normally cleared by a relatively rapid clearance process. In addition, we cannot rule out a small contribution from L-type VGCCs during the second of paired pulses, since there was a small reduction in the overall enhancement of the $\Delta R2 \Delta F/F_0$ in the presence of nimodipine (Fig. 5C).

The NCX is a high capacity, low affinity Ca^{2+} transporter with potential importance in $[\text{Ca}^{2+}]_{\text{pre}}$ clearance because it extrudes Ca^{2+} rather than buffering or sequestering it within the presynaptic terminal. Recent ultrastructural studies show only a minor involvement of NCX1-3 in presynaptic terminals [56] and studies using NCKX2 knockout mice indicate that this

exchanger does not significantly contribute to PPF [57]. Our experiments with benzamil demonstrate that NCX contributes neither to the difference between P1 $\Delta F/F_0$ and $\Delta R2 \Delta F/F_0$ (Fig. 5D) nor to the generation of PPF (Fig. 6B).

The PMCA has a high affinity for Ca^{2+} and is thus a good candidate for rapid $[\text{Ca}^{2+}]_{\text{pre}}$ clearance [58]. Our attempts to block the PMCA with carboxyeosin were complicated by two factors. First, it was necessary to measure $[\text{Ca}^{2+}]_{\text{res}}$ with Fura-FF instead of Mg-Green and this forced us to fit the $\Delta F/F_0$ decay data with a single instead of the double exponential used in all of the other experiments. Second, to minimize potential complications from block of the SERCA pump by carboxyeosin, we carried out these experiments in the presence of cyclopiazonic acid, which had the expected result of increasing P1 $\Delta F/F_0$ to approximately match $\Delta R2 \Delta F/F_0$. We did not, however, observe any additional increase in the decay with carboxyeosin, although this effect could have been occluded by the action of cyclopiazonic acid. We interpret these results therefore to indicate that PMCA is not the major factor in clearing $[\text{Ca}^{2+}]_{\text{res}}$ after a single pulse.

4.4. Contribution of mitochondria to $[\text{Ca}^{2+}]_{\text{res}}$

Assessment of the role of the MCU in $[\text{Ca}^{2+}]_{\text{res}}$ clearance is complicated by the role of mitochondria in ATP production and the ATP requirement for other Ca^{2+} clearance pathways. Because mitochondria are a low affinity, high capacity Ca^{2+} sequestration organelle, the MCU is unlikely to make an important contribution to the clearance of $[\text{Ca}^{2+}]_{\text{res}}$ during pairs of pulses in Schaffer collateral synaptic boutons [59]. Nevertheless, because mitochondria are present in presynaptic terminals, we performed several experiments to address whether a mitochondria-dependent processes could influence Ca^{2+} dynamics during short-term synaptic plasticity.

Ruthenium red has been used to block the MCU [60], but this molecule is rather membrane impermeant and so to be most effective it requires intracellular injection [61], which was not possible in our experiments. Mn^{2+} ions, which have been reported to block the MCU after successfully entering the cytoplasm, are inconsistent in their reported effectiveness in blocking mitochondrial Ca^{2+} uptake [62]. We thus decided to use the indirect approach of abolishing $\Delta\psi_m$ through application of the protonophore FCCP, which then prevents mitochondrial Ca^{2+} uptake by the $\Delta\psi_m$ -dependent MCU [33]. We found unfortunately that FCCP produced a significant decrease in the P1 $\Delta F/F_0$, the $\Delta R2 \Delta F/F_0$, and the postsynaptic fEPSP, which thereby precluded its use to assess the role of the MCU in $[\text{Ca}^{2+}]_{\text{res}}$ clearance. Abolishing $\Delta\psi_m$ introduces the additional complication of depleting cytoplasmic ATP stores and thereby influencing other cytoplasmic ATP-dependent processes. We took two approaches to minimize this possible complication. First, we used oligomycin, a blocker of the F_0 portion of the mitochondrial H^+ -ATP-synthase, which blocks both its ATP synthesis activity and its reverse mode ATP hydrolysis activity [63]. Second, we kept the FCCP exposure short such that all $\Delta F/F_0$ measurements were made between 3 min, when there was about 92% bath exchange, and 6 min, when there was over 99% bath exchange. Unexpectedly, oligomycin eliminated the increase in $\Delta R2 \Delta F/F_0$ as a result of a decrease in the relative contribution of the slow component to the overall $\Delta F/F_0$ decay. One possible explanation for this result is that in the presence of glucose, oligomycin does not cause cytoplasmic [ATP] to fall below that required for SERCA function [64]. However, it has been suggested that mitochondria produce microdomains of ATP that favor SERCA-mediated Ca^{2+} clearance [65] and this is consistent with electron microscopic evidence for very close apposition of mitochondria and ER [66]. Thus, the decrease in the relative contribution of the slow component of $\Delta F/F_0$ clearance could be caused by oligomycin reducing the effectiveness of a privileged mitochondrial ATP source for SERCA. As a result, SERCA clearance would be reduced, but not eliminated, and other faster Ca^{2+} clearance pathways would make a more significant contribution. Importantly, however, in the presence of oligomycin or FCCP with oligomycin, the τ_s of $\Delta R2 \Delta F/F_0$ is

significantly slower than that of R1 $\Delta F/F_0$ (Fig. 5F1), which we interpret to indicate that the MCU is not a major contributor to $[Ca^{2+}]_{res}$ clearance during paired pulses.

4.5. Modeling $[Ca^{2+}]_{res}$ kinetics

The kinetics of presynaptic $[Ca^{2+}]_{res}$ clearance are an important component of the timecourse of short-term synaptic plasticity [67] and we have shown selective involvement of specific clearance pathways on PPF (Fig. 6B). Importantly, the $[Ca^{2+}]_{res}$ decays in this study were best fit with a double exponential, which is consistent with a cooperative mechanism such as SERCA-dependent uptake into the ER. ER Ca^{2+} stores, while only minimally filled in the basal state, are capable of significant Ca^{2+} sequestration (Fig. 3) and block of SERCA pumps significantly decreases the τ_s of $[Ca^{2+}]_{res}$ clearance (Fig. 4). We hypothesize from these observations that the SERCA-dependent sequestration into the ER plays a major role in presynaptic $[Ca^{2+}]_{res}$ clearance and that a rate-limiting step in this pathway is responsible for the increased τ_s during R2. Lacking the ability to measure ER Ca^{2+} kinetics in presynaptic terminals without membrane permeabilization [68], we resorted to a reduced kinetic model to predict SERCA kinetics based on fits to our paired pulse $[Ca^{2+}]_{res}$ data (see Appendix for details). While a wide range of initial parameters fit the $[Ca^{2+}]_{res}$ decay during R1, the observed increase in τ_s during R2 is most consistently predicted by a reduction of the forward rate constant, k_4 , for cytoplasmic Ca^{2+} binding to SERCA (Fig. 7A). Additionally, when we used parameters from pairs of pulses to fit to $[Ca^{2+}]_{res}$ data from 5 pulses, the model predicted that the free [SERCA] continued to decline and the ER buffered Ca^{2+} concentration ($[CaB]$) rose, but did not saturate (Fig. 7D), which is consistent with our observation that the ER has the capacity to accumulate Ca^{2+} during multiple pulses (Fig. 3) with potential repercussions in the determination of synaptic frequency response.

4.6. Physiological roles of SERCA Ca^{2+} uptake

Because of the close dependency of short-term plasticity and working memory on $[Ca^{2+}]_{res}$, it is important to understand the mechanisms by which Ca^{2+} is cleared from the cytoplasm of the presynaptic terminal between repetitive action potentials. As we have shown here, Ca^{2+} sequestered into and released from the ER, and the kinetics and magnitude of this process have important consequences in determining the Ca^{2+} dynamics of this specialized cellular compartment. This clearance process is but one adaptation of a system that underlies important physiological properties of many cells in which Ca^{2+} dynamics vary not only from cell to cell but even among cellular compartments. For example, in pancreatic acinar cells, the kinetics of transmembrane flux are relatively slow so sequestration into and release from the ER greatly impact $[Ca^{2+}]_i$. Of special importance in acinar cells, feedback between sequestration and release produces Ca^{2+} waves whose oscillatory period is determined by the density of SERCA [69]. In skeletal muscle and to a lesser degree in cardiac muscle, the transmembrane flux is small and again uptake into and release from the sarcoplasmic reticulum is the major factor that controls sarcoplasmic $[Ca^{2+}]_i$. In this instance, release from the sarcoplasmic reticulum is rapidly triggered by voltage or Ca^{2+} while uptake into the sarcoplasmic reticulum by SERCA determines the timecourse of muscle relaxation. Thus specific SERCA isoforms can influence muscle twitch speed [70] and the level of SERCA activity plays a central role in frequency-dependent contractile force development [71]. In contrast to these non-neuronal cells, in the basal state, there appears to be very little releasable Ca^{2+} in Ca^{2+} -sensitive ER stores of Schaffer collateral presynaptic terminals, although these stores have considerable capacity to sequester $[Ca^{2+}]_i$. The persistence of $[Ca^{2+}]_i$ in presynaptic terminals is more transient than in the non-neuronal cells described above, but, on the timescale of short-term synaptic plasticity, SERCA transport into the ER is still a major clearance pathway. Furthermore, we have predicted that the cytoplasmic Ca^{2+} binding sites of the SERCA pump are easily saturated in presynaptic terminals such that after only a few action potentials this clearance pathway is limited by SERCA turnover, although not by the Ca^{2+} storage capacity of the ER. As in the non-neuronal

cells, SERCA properties have important physiological consequences that, in the case of presynaptic terminals, are a leading determinant of the magnitude short-term synaptic plasticity.

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Appendix A

The double exponential decay, which best fit our $[Ca^{2+}]_{res}$ data, is consistent with either a sequential process or two parallel kinetic processes that occur simultaneously acting on separate pools. Because it was indicated by our experimental observations, we tested whether a simplified SERCA model, using the range of published parameters, could predict the observed double exponential decay kinetics. The resultant kinetic model accurately predicted both the fast and slow components of decay as a consequence of a single sequential process. This simplified model was limited to short trains of pulses during which there is a limited rise in basal $[Ca^{2+}]_{pre}$. More significant increases in $[Ca^{2+}]_{pre}$ would be expected to activate additional mechanisms such as plasma membrane transport or mitochondrial uptake.

Kinetic modeling of presynaptic $[Ca^{2+}]_{res}$ used a reduced state diagram and was analyzed in a differential form that included k_4 , k_{-4} , k_{-4*} , k_{4*} , k_{BER} , k_{-BER} , k_3 (see Fig. 7A and Eqs. (4)–(14) below). Presynaptic Ca^{2+} influx was modeled as a Gaussian process with a half width of 2.5ms that was adjusted to fit the derivative of the data from Fig. 1A1 scaled to reflect an estimated peak of 500 nM. $[Ca^{2+}]_{res}$ clearance was evaluated using ordinary differential equations (ODE) and initial conditions (ICs) in a Matlab program with ode45 4th or 5th order Runge-Kutta numerical integration. The non-predefined ICs (see table) were then fit to the data using a nonlinear least squares analysis, after using Potterswheel [35] to determine component sensitivity for all parameters. Parameters found in the literature, but highly sensitive to the fit, were allowed to vary over a pre-specified range (either restricted to the range specified in the literature or held to an order of magnitude, see Table 1). First the $[Ca^{2+}]_{res}$ data for paired pulses from Fig. 1A1 were fit ($R^2 = 0.95$) and the resultant parameters were then applied to a single pulse model, which was compared with single pulse data from the same experiment ($R^2 = 0.93$). Next the same parameter range was used to fit an additional 23 paired pulse $[Ca^{2+}]_{res}$ data sets ($R^2 = 0.86$). Finally, we fit $[Ca^{2+}]_{res}$ data from a separate experiment with 5 pulses at 50ms interpulse interval ($R^2 = 0.94$). In this instance the same parameter range was used except that the ryanodine receptor-dependent efflux from the ER was increased to reflect an expected larger Ca^{2+} -dependent component.

Assumptions for Model

1. Primary $[Ca^{2+}]_{cyt}$ clearance is a receptor-dependent process with lumped passive kinetics approximated by the $\Delta F/F_0$ decay in Ba^{2+} (Fig. 2C) and that of the second pulse in ACSF (Fig. 1C).
2. Receptor-dependent $[Ca^{2+}]_{cyt}$ clearance is primarily through SERCA uptake into the ER (Fig. 4).
3. In the basal state, there is a low releasable $[Ca^{2+}]_{ER}$, but the ER is capable of considerable Ca^{2+} uptake (Fig. 3).

4. All concentrations and rates that were not fit to the data, were taken from published values (see Table 2).

$$r_3 = -k_3[\text{Ca}]_{\text{ER}} \quad (4)$$

$$r_4 = -k_4 \left([\text{Ca}]^2 [\text{SERCA}] - [\text{CaSERCA}] K d_4 \right) \quad (5)$$

$$r_{4*} = -k_{4*} \left([\text{CaSERCA}] - \frac{[\text{Ca}]_{\text{ER}}^2 [\text{SERCA}]}{K d_{4*}} \right) \quad (6)$$

$$r_{\text{BERC}} = -k_{\text{BER}} \left([\text{Ca}]_{\text{ER}} [\text{B}]_{\text{ER}} - [\text{Ca}]_{\text{ER}} K d_{4*} \right) \quad (7)$$

$$r_{\text{barium}} = -k_{\text{barium}} [\text{Ca}] \quad (8)$$

$$r_{\text{stimulated}} = 500 e^{-2(t-t_{\text{stim}})^2} \quad (9)$$

$$\frac{d[\text{Ca}]}{dt} = 2r_4 - r_3 \gamma + r_{\text{barium}} + r_{\text{stimulated}} \quad (10)$$

$$\frac{d[\text{SERCA}]}{dt} = r_4 - r_{4*} \quad (11)$$

$$\frac{d[\text{CaSERCA}]}{dt} = -r_4 + r_{4*} \quad (12)$$

$$\frac{d[\text{Ca}]_{\text{ER}}}{dt} = -\frac{2r_{4*}}{\gamma} + r_{\text{BERC}} + r_3 \quad (13)$$

$$\frac{d[\text{B}]_{\text{ER}}}{dt} = r_{\text{BERC}} \quad (14)$$

$$\frac{d[\text{CaB}]_{\text{ER}}}{dt} = -r_{\text{BERC}} \quad (15)$$

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Fig. 1. Presynaptic $[Ca^{2+}]_i$ response to paired pulses. (A1). Single pulse (black) and paired pulses (gray) at 50ms IPI. (A2). Single pulse $\Delta F/F_0$ (black) and $\Delta R2 \Delta F/F_0$ (gray) with superimposed $\int \Delta F/F_0$. (B) Average P1 $\int \Delta F/F_0$ (black) and $\Delta R2 \int \Delta F/F_0$ (gray) for ACSF controls. (Paired t -test, $n = 44$). (C) Average slow decay time constant, τ_s , for P1 $\Delta F/F_0$ (black) and $\Delta R2 \Delta F/F_0$ (gray) for ACSF controls (Paired t -test, $n = 27$, Mg-Green).

Fig. 2.

Barium blocks postsynaptic fEPSPs and slows $\Delta F/F_0$ decay (A) *Top*: fEPSP normalized to initial value of R1 ACSF; R1 (black); R2 (gray); *Bottom*: $\% \Delta F/F_0$ R1 (black); ΔR_2 (gray). (B) Representative P1 $\% \Delta F/F_0$ decay traces (gray) with double exponential fit using equation 1 (black). *Top*: ACSF, middle, Ba^{2+} ACSF, bottom, wash. (C) Summary of parameters from double exponential fit to P1 $\Delta F/F_0$ decay. *Top*: τ_s . *Bottom*: % fast component calculated as $100 \times B$ in Eq. (1). There was also a significant decrease in the initial value of $\Delta F/F_0$ (A in Eq. (1)) in Ba^{2+} , $p < 0.005$; but no change in τ_f , $p = 0.595$ (not shown). (One-way ANOVA, *Top*: $F_{2,27} = 10.507$, *Bottom*: $F_{2,27} = 9.816$, Bonferroni post hoc, $n = 10$). (D) $\Delta R_2 [\Delta F/F_0]$ in ACSF and in Ba^{2+} ACSF. (Amplitudes of the Ca^{2+} and Ba^{2+} $\Delta F/F_0$ signals cannot be compared directly so each was normalized to its respective R1 $\Delta F/F_0$.) (One-sample t -test against theoretical mean of 1.0, Ba^{2+} $p = 0.23$, $n = 4$). (E) Arrhenius plot of $\ln (\Delta R_2 [\Delta F/F_0] / P1 [\Delta F/F_0])$ vs. $1/\text{temperature}$ for 7 slices each measured at 4 temperatures. Slopes, m , were used to calculate Q_{10} as: $Q_{10} = e^{[-10m/T_1(T_1+10)]}$. Average $Q_{10} = 2.94 \pm 0.64$. ($p < 0.05$ by one sample t -test against a theoretical mean of 1.3 which is the minimum expected value for an active process, $n = 7$, Mg-Green).

Fig. 3.

Filling state of presynaptic Ca^{2+} stores. (A) Bath application of $3\mu M$ thapsigargin. Response to $40mM$ K^+ was used as an indication of successful Ca^{2+} responses in all experiments in this Figure, but is shown only in this panel. ($n = 5$). (B) Bath application of $20mM$ caffeine with no preload (black filled circles, $n = 6$) or bath application of $10mM$ caffeine following 30 s preload in $40mM$ K^+ (gray filled squares, $n = 6$). Comparing the first and the last 1min periods between the preloaded and no preloaded conditions with ANOVA ($F_{3,12} = 187.69$, Bonferroni post hoc) showed $p = 0.169$ for the first period in ACSF and $p < 0.001$ for the last period in 10 or $20mM$ caffeine. Responses to $40mM$ K^+ preload, but without $10mM$ caffeine (not shown) were indistinguishable from no preload condition. ($n=3$) (C) Bath application of $10mM$ caffeine following high frequency stimulation at vertical arrow: $2 \times 20 @ 500$ Hz then caffeine (gray filled squares, $n = 6$); $2 \times 20 @ 500$ Hz no caffeine (black open squares, $n = 4$); $2 \times 20 @ 100$ Hz then caffeine (gray open circles, $n = 6$); no stim then caffeine (black filled circles, $n = 12$). Comparing the first and the last 1minute periods of the $2 \times 20 @ 500$ Hz records between caffeine and no caffeine with ANOVA ($F_{3,12} = 601.43$, Bonferroni post hoc) showed $p = 0.891$ for caffeine vs. no caffeine in the first period and $p < 0.001$ for these two conditions in the last period. (Fura-2 ratio images ($350/380$ nm) were taken every 15 s.).

Fig. 4. Block of SERCA pumps increases P1 $\int \Delta F/F_0$. (A) Representative $\% \Delta F/F_0$ and $\int \% \Delta F/F_0$ traces, (A1) ACSF, P1 (black); $\Delta R2$ (gray), (A2) 3 μ M thapsigargin, P1 (black); $\Delta R2$ (gray). ($\int \% \Delta F/F_0$ scale units are: $\% \Delta F/F_0 \times \text{ms}$) (B) Maximum $\int \Delta F/F_0$ following paired pulses in 3 μ M thapsigargin normalized to maximum P1 $\int \Delta F/F_0$ in ACSF. (One-sample t-test against theoretical mean of 1.0, $n = 10$.) (C) τ_s for fits to equation 1 following a single pulse in 3 μ M thapsigargin. In addition there was no significant difference in: peak $\% \Delta F/F_0$, $p = 0.183$; τ_f , $p = 0.254$; or $\% \tau_f$, $p = 0.858$ (not shown). (Paired t -test, $n = 10$, (Mg-Green.).

Fig. 5.

Contribution of plasma membrane Ca^{2+} flux to $[\text{Ca}^{2+}]_{\text{pre}}$ (A and B) Representative $\% \Delta F/F_0$ and $\int \% \Delta F/F_0$ traces (P1, black; $\Delta R2$, gray). (A1) 10 μM nimodipine, (A2) 10 μM nimodipine + 100 μM benzamil, (B1) 10 μM cyclopiazonic acid, (B2). 10 μM cyclopiazonic acid + 40 μM carboxyeosine. (C) Peak $\int \Delta F/F_0$ normalized to ACSF P1 $\int \Delta F/F_0$ following paired pulses in 10 μM nimodipine. (One-sample t -test against theoretical mean of 1.0, P1 nimodipine $p = 0.28$, $n = 11$, Mg Green). (D) Maximum $\int \Delta F/F_0$ following paired pulses in 100 μM benzamil in the presence of 10 μM nimodipine normalized to maximum P1 $\int \Delta F/F_0$ in ACSF for the same slice. (One-sample t -test against theoretical mean of 1.0, P1 nimodipine $p = 0.27$, P1 nimodipine + benzamil $p = 0.45$, $n = 11$, Mg-Green) (E) Peak $\int \Delta F/F_0$ normalized to P1 $\int \Delta F/F_0$ in ACSF for each slice following paired pulses in 40 μM carboxyeosine in the presence of 10 μM cyclopiazonic acid. ($\Delta R2$ $\int \Delta F/F_0$ in ACSF was included for comparison.) (One-sample t -test against theoretical mean of 1.0, $n = 7$, Fura-FF) (F) $[\text{Ca}^{2+}]_{\text{res}}$ timecourse in ACSF ($n = 19$), 2.5 $\mu\text{g}/\text{ml}$ oligomycin ($n = 10$), and following subsequent addition of 1 μM FCCP ($n = 16$) (F1). τ_s for $[\text{Ca}^{2+}]_{\text{res}}$ decay for P1 $\Delta F/F_0$ and $\Delta R2$ $\Delta F/F_0$ decay (One-way ANOVA, $F_{5,84} = 10.14$ with Bonferroni post hoc, Mg Green). (F2) Percent slow component of $[\text{Ca}^{2+}]_{\text{res}}$ decay calculated as $100 \times A(1-B)$ from Eq. (1) (One-sample t -test against theoretical mean of 100%) (F3). τ_f for $[\text{Ca}^{2+}]_{\text{res}}$ decay for P1 $\Delta F/F_0$ and $\Delta R2$ $\Delta F/F_0$ decay. There was no significant of drug on τ_f . One-way ANOVA, $F_{5,78} = 0.9787$, $P = 0.4362$).

Fig. 6. Effect of $[Ca^{2+}]_{res}$ clearance pathways on paired pulse facilitation. (A1) PPF at 50ms interpulse interval in ACSF. (A2) PPF in the same slice as A1 following a 15 min exposure to 3μM thapsigargin. (Stimulus artifacts have been truncated for clarity.) (B) Average PPF in ACSF ($n = 41$), 3μM thapsigargin (TGN, $n = 17$), 40μM carboxyeosine plus 10μM cyclopiazonic acid (CE-CPA, $n = 11$), 10μM nimodipine plus 100μM benzamil (nim-benz, $n = 20$), 2.5μg/ml oligomycin plus 1μM FCCP (oligo-FCCP, $n = 25$). (One-way ANOVA, $F_{4,110} = 1.965$ with Bonferroni post hoc.).

Fig. 7. Ca^{2+} clearance kinetics. (A) Ca^{2+} clearance pathways under consideration in this study 1. VGCC, 2. buffering (B) and diffusion, 3. RyR, 4. SERCA, 5a. PMCA, 5b NCX, 6. MCU (see Section 1). Pathways indicated in bold are included in the kinetic model. (B) Representative trace from Fig. 1A1 fit with the reduced model including only buffer and SERCA/ER components ($R^2 = 0.95$). (C) Average data from fits to 23 $[\text{Ca}^{2+}]_{\text{res}}$ traces in ACSF. Black, predicted $[\text{Ca}^{2+}]_{\text{cyt}}$; gray, predicted free $[\text{SERCA}]$. (D) *Top panel*: fit to $[\text{Ca}^{2+}]_{\text{res}}$ during a train of 5 pulses at 50ms IPI ($R^2 = 0.94$). *Middle panel*: predicted free cytoplasmic SERCA Ca^{2+} binding sites. *Bottom panel*: predicted change of ER buffered Ca^{2+} .

Table 1

Parameters that varied during kinetic modeling. Model value is the final value obtained after fitting. Published value in cited reference for the same parameter.

Model parameter	Model value	Published values	Reference	Cell type	
[Ca]	133	nM	95	nM [72]	Hela
[SERC A]	1062	nM	15 (varied)	$\mu\text{mol/L cyt}$ [69]	Non-excitable
[CaSERC A]	3	nM			
[CaB] _{ER}	0.120	μM	10	μM [72]	Hela
$k_{4^{**}}$	0.92	ms^{-1}	0.4	s^{-1} [69]	Non-excitable
			600	s^{-1} [73]	Human SERCA
k_4	1.097E-07	$\text{nM}^{-1} \text{ms}^{-1}$	0.9	$\mu\text{M}^{-1} \text{s}^{-1}$ [74]	Non-excitable
			67	$\text{nM}^{-1} \text{s}^{-1}$ [75]	PC 12
k_3	0.0009	ms^{-1}	2	s^{-1} [76]	Non-excitable
[Ca] _{ER}	40	nM	low		Fig. 2
k_{barium}	0.0069	ms^{-1}	0.002	ms^{-1} Fig. 3	

Table 2

Parameters from cited reference that were fixed in kinetic model.

Model parameter	Model value		Reference	Cell type
$[B]_{ER}$	375000	nmol/L ER	[69]	Non-excitable
Kd_4	700000	(nmol/L cyt) ²	[69]	Non-excitable
Kd_{4*}	10	(nmol/L ER) ²	[69]	Non-excitable
k_{-BER}	0.08	ms ⁻¹	[69]	Non-excitable
k_{BER}	0.000001	((nmol/L ER) ⁻¹ ms ⁻¹)	[69]	Non-excitable
γ	0.185	Vol _{ER} /Vol _{Cyt}	[69]	Non-excitable
Kd_{BER}	80000	nmol/L ER		