Inhibition of Mitochondrial Ca\textsuperscript{2+} Uptake Affects Phasic Release From Motor Terminals Differently Depending on External [Ca\textsuperscript{2+}]

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Inhibition of mitochondrial Ca\textsuperscript{2+} uptake affects phasic release from motor terminals differently depending on external [Ca\textsuperscript{2+}]. J. Neurophysiol 90: 491–502, 2003. First published April 2, 2003; 10.1152/jn.00012.2003. We investigated how inhibition of mitochondrial Ca\textsuperscript{2+} uptake affects stimulation-induced increases in cytosolic [Ca\textsuperscript{2+}] and phasic and asynchronous transmitter release in lizard motor terminals in 2 and 0.5 mM bath [Ca\textsuperscript{2+}]. Lowering bath [Ca\textsuperscript{2+}] reduced the rate of rise, but not the final amplitude, of the increase in mitochondrial Ca\textsuperscript{2+} during 50-Hz stimulation. The amplitude of the stimulation-induced increase in cytosolic [Ca\textsuperscript{2+}] was reduced in low-bath [Ca\textsuperscript{2+}] and increased when mitochondrial Ca\textsuperscript{2+} uptake was inhibited by depolarizing mitochondria. In 2 mM Ca\textsuperscript{2+}, end-plate potentials (epps) depressed by 53% after 10 s of 50-Hz stimulation, and this depression increased to 80% after mitochondrial depolarization. In contrast, in 0.5 mM Ca\textsuperscript{2+} the same stimulation pattern increased epps by ~3.4-fold, and this increase was even greater (transiently) after mitochondrial depolarization. In both 2 and 0.5 mM [Ca\textsuperscript{2+}], mitochondrial depolarization increased asynchronous release during the 50-Hz train and increased the total vesicular release (phasic and asynchronous) measured by destaining of the styryl dye FM2-10. These results suggest that by limiting the stimulation-induced increase in cytosolic [Ca\textsuperscript{2+}], mitochondrial Ca\textsuperscript{2+} uptake maintains a high ratio of phasic to asynchronous release, thus helping to sustain neuromuscular transmission during repetitive stimulation.

INTRODUCTION

Reversible sequestration of Ca\textsuperscript{2+} by mitochondria is important for managing moderate-to-large cytosolic Ca\textsuperscript{2+} loads in the somata of neurons and other secretory cells (Babcock and Hille 1998; Colegrove et al. 2000a,b; Friel and Tsien 1994; Thayer and Miller 1990; Werth and Thayer 1994). Mitochondria take up Ca\textsuperscript{2+} passively via a uniporter, down the electrochemical gradient across the inner mitochondrial membrane. Most of this gradient is attributable to the membrane potential gradient \( \Psi_m \) (~150 to ~200 mV), created by H\textsuperscript{+} extrusion via the complexes of the electron transport chain (reviewed in Gunter and Gunter 1994; Gunter and Pfeiffer 1990). In some cells, this mitochondrial Ca\textsuperscript{2+} uptake begins at \( \leq 300 \) mM cytosolic [Ca\textsuperscript{2+}] (Babcock et al. 1997; Colegrove et al. 2000a; David et al. 1998). Mitochondrial Ca\textsuperscript{2+} uptake appears to be especially important in nerve terminals, which contain abundant mitochondria and experience a large Ca\textsuperscript{2+} influx during activity. During repetitive action potential discharge, cytosolic [Ca\textsuperscript{2+}] in frog, crayfish, lizard, and mouse motor terminals increases at first rapidly, then more slowly (David et al. 1998; David and Barrett 2000; Suzuki et al. 2000, 2002; Tang and Zucker 1997; Wu and Betz 1996). These studies showed that when mitochondrial Ca\textsuperscript{2+} uptake is blocked, cytosolic [Ca\textsuperscript{2+}] continues to increase rapidly throughout the stimulus train, reaching much higher final values. Steunkel (1994) demonstrated similar results in rat neurohypophysis depolarized using voltage-clamp pulses.

The present study investigated how inhibition of mitochondrial Ca\textsuperscript{2+} uptake affects transmitter release from lizard motor terminals. Previous studies showed that \( \Psi_m \) depolarizing agents increase the asynchronous release recorded in physiological saline, Ca\textsuperscript{2+}-free saline, and media containing high [K\textsuperscript{+}] (Alnaes and Rahamimoff 1975; Calupca et al. 2001; Molgo and Pecot-Dechavassine 1988; Washio 1982). In low bath [Ca\textsuperscript{2+}], \( \Psi_m \)-depolarizing agents increased the rate at which end-plate potential (epp) amplitudes increased during 20-Hz stimulation of frog motor terminals (Zengel et al. 1994). In contrast, in physiological Ca\textsuperscript{2+} \( \Psi_m \) depolarization accelerated the depression of epp amplitudes in mouse motor terminals (David and Barrett 2003). We measured total vesicular release using destaining of FM2-10, and phasic and asynchronous quantal release using electrophysiological techniques, in lizard motor terminals in both low (0.5 mM) and physiological (2 mM) bath [Ca\textsuperscript{2+}]. We report that \( \Psi_m \) depolarization increased total vesicular release and asynchronous quantal release during 50-Hz stimulation in both bath [Ca\textsuperscript{2+}]. However, the effects of \( \Psi_m \) depolarization on phasic release during repetitive stimulation were markedly different, with a transient increase in 0.5 mM Ca\textsuperscript{2+} but an accelerated depression in physiological [Ca\textsuperscript{2+}]. An unexpected finding was that epp quantal contents in 0.5 and 2 mM Ca\textsuperscript{2+} converged to a common value during repetitive stimulation.

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**METHODS**

**Preparation**

Neuromuscular preparations from external intercostal muscles were dissected from the lizard Anolis sagrei. Animals were decapitated and pithed after euthanasia in 100% CO2. The preparation was mounted in a silicon chamber constructed on a No. 1 glass coverslip and bathed in normal lizard saline (NLS, pH = 7.4) composed of (mM) 157 NaCl, 4 KCl, 2 CaCl2, 2 MgCl2, 5.5 glucose, and 1 HEPES buffer. In some experiments, bath [Ca2+] was reduced to 0.5 mM. Multiple muscles (4–6), each innervated by its own motor nerve, were accessible in each preparation. All experiments were performed at room temperature.

Action potentials were evoked in the motor nerve via a suction electrode by applying brief (0.3 ms) suprathreshold depolarizing pulses. Nerve stimulation was applied at 1 Hz throughout the experiment, with trains of 50 Hz for 10 s (or sometimes 50 s) superimposed. Intervals between stimulus trains were ≥10 min to ensure complete recovery of mitochondrial [Ca2+].

**Imaging changes in cytosolic and mitochondrial [Ca2+]**

Measurements of stimulation-induced changes in cytosolic [Ca2+] were made using the Ca2+ indicator Oregon Green BAPTA 5N (OG-5N), injected ionophoretically via a micropipette inserted into the motor axon (David et al. 1997). OG-5N was excited at 488 nm and emitted light collected at >515 nm. The low affinity of OG-5N (Kd ~ 40–60 μM) ensured minimal disruption of cellular Ca2+-dependent processes and prevented dye saturation at the higher cytosolic [Ca2+] attained during repetitive stimulation with Ψm depolarization.

Rhod dyes were used to monitor changes in mitochondrial [Ca2+]. Rhod-2 (Kd ~ 0.5 μM) or rhod-5F (Kd ~ 1.9 μM) were targeted into the mitochondria by bath-loading 50 μg/ml of the acetoxyxymester (AM) form for ≥2 min, followed by washout via 5–10 bath exchanges of NLS for 1 h. Rhod dyes were excited at 528 nm, and emission was monitored at >570 nm. Mitochondrial localization was confirmed using the following morphological, pharmacological, and kinetic criteria. First, the fluorescence increase following trains of stimuli was punctate and localized within the nerve terminal (not the axon), consistent with the known clustering of mitochondria within motor terminals. Second, the stimulation-induced increase in fluorescence was abolished by Ψm depolarizing agents (Fig. 1A). Third, the rate of rise of mitochondrial [Ca2+] was slower than that of cytosolic [Ca2+], and when stimulation ceased, mitochondrial [Ca2+] decayed slowly, without the rapid initial component characteristic of cytosolic [Ca2+].

Terms were imaged with a confocal laser-scanning microscope (Nikon ×40 water-immersion lens or a Nikon ×20 air lens). Laser power was kept low to minimize photodamage. Data were collected using an Indy workstation (Silicon Graphics) with Noran InterVision software. Images were sampled at a rate of 0.533 s/frame and analyzed using IMAGE+ software (version 4.0, Digital Micro Opts, Auckland, NZ). Regions of interest (ROIs) were drawn around the fluorescent terminals and the same ROIs were used across all images collected for a given terminal. Background staining intensity was averaged from ROIs drawn outside of and adjacent to the boutons. Fluorescence was reported as ΔF/F, calculated as

\[
\Delta F/F = (F - F_{rest})/(F_{rest} - B)
\]

where \(F\) is the measured fluorescence intensity, \(F_{rest}\) is the resting (prestimulus) fluorescence, and \(B\) is the background signal. Stimulation-induced OG-5N responses were converted to estimates of cyto-
Electrophysiological measurements

For these recordings, we needed to block muscle contractions in a manner that permitted recording of both phasic and asynchronous release. The long duration of the recordings (multiple 50–Hz trains separated by ≥10-min intervals) precluded use of voltage clamping or focal extracellular recording. A toxin (μ-conotoxin GIIIB) that blocks muscle but not nerve Na⁺ channels in frogs and mice does not have this selective effect in lizard preparations. Thus we used two other approaches to reduce the detrimental effect of muscle contractions on intracellular recordings. First we selected fibers in which electrode damage had partially depolarized the resting potential to −40 to −50 mV for recordings in 2 mM Ca²⁺ or to −60 to −70 mV for recordings in 0.5 mM Ca²⁺ (see Table 1). This depolarization increased the threshold for action potential generation and thus minimized contractions. Second, we used a floating microelectrode (Woodbury and Brady 1956), manufactured by wrapping the middle cm of a 3-cm silver wire electrode around a −0.2-mm-diam rod to create a small spring. One end of the silver wire was pushed into the barrel of a microelectrode and secured with a small piece of modeling clay. The other end was mounted on the micromanipulator. Microelectrodes (8–20 MΩ) were pulled from borosilicate glass and filled with 3 M KCl. Data were collected using an Axoclamp 2A preamplifier (Axon Instruments, Union City CA), using Axoclamp 8.1 software.

Epp amplitudes were measured using Clampfit software (Axon Instruments). These amplitudes were then corrected using an equation that combines Martin’s (1976, Eq. 6) correction for nonlinear summation, and Kelly’s (1978, Eq. 1) correction to a common resting potential, to facilitate comparison of epp amplitudes recorded from fibers with different resting potentials. Martin’s (1976) formula can be written as

\[
epp_c = epp/(1 - f_{epp}(V_r - V_e))
\]

where \(epp_c\) is the corrected epp amplitude, \(epp\), is the peak amplitude of the recorded epp, \(f\) is an empirical correction factor (see following text) introduced to prevent over-correction of epp amplitude, \(V_r\) is the recorded resting muscle membrane potential, and \(V_e\) is the reversal potential of the epp (estimated to be −10 mV), Kelly’s (1978) correction factor is \((V_r - V_e)/(V_r - V_e)\), where \(V_e\) is the selected common resting potential (see following text). Combining Martin’s formula with Kelly’s correction yields

\[
epp_c = \frac{epp}{1 - f_{epp}(V_r - V_e)}
\]

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<th>TABLE 1. Effects of treatments on pretrain values</th>
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Values are means ± SE for 6–9 end-plates in 2 and 0.5 mM Ca²⁺. At each end-plate, averages were calculated from 15 to 60 epps (evoked at 1 Hz) and from 20 to 160 mepps. Oligo = oligomycin. Depolarization of the membrane potential across the inner mitochondrial membrane (\(\Psi_m\)) was achieved by addition of 1 μM carbonyl cyanide m-chlorophenyl hydrazone (CCCP) or 2 μM antimycin A1. Epp and mepp amplitudes are given before (uncorrected) and after (corrected) corrections for non-linear summation and resting membrane potential (Eq. 4 in METHODS). For 2 mM Ca²⁺, mepp amplitudes (uncorrected) with \(\Psi_m\) depolarization were significantly different from control (\(P < 0.05\), Student-Newman-Keuls test), but this difference failed to reach significance after these corrections. Calupca et al. (2001) also found no effect of \(\Psi_m\) depolarization on mepp frequency in resting snake motor terminals. The depolarized resting membrane potentials (especially in 2 mM Ca²⁺) were intentional, produced by the recording microelectrode (see METHODS).
To mitochondrial localization of AM-loaded rhod dyes. Figure 1B compares rhod-2 fluorescence changes of ionophoretically-injected OG-5N, assuming a resting level of 0.1 μM.}

\[
\text{epp} = \frac{\text{pp} - \text{epp} \times (V_c - V)}{(V_c - V)(V - V_c)}.
\]

The empirical factor \( f \) was set to 0.2, by recording epps and miniature epps (mepps) from muscle fibers whose resting potential changed over time and determining the value of \( f \) that yielded similar values for the quantal content at different resting potentials. \( V_c \) was set to −60 mV, approximating the lower range of resting potentials recorded in 0.5 mM Ca\(^{2+}\); the exact value used for \( V_c \) would not affect any of the conclusions presented here. Epps that triggered action potentials were excluded from this analysis.

Mepps were detected and analyzed using Mini Analysis Program (version 5.3.5, Synaptosoft, Jenin Software, Leonia NJ) with visual inspection of recordings to exclude noise and stimulus artifacts. To estimate mepp frequency during the interstimulus interval, we counted mepps during the last 10 ms of the 20-ms interstimulus interval and multiplied by 2 to estimate the total number of mepps in the interstimulus interval. However, the decay of large epps sometimes extended over most of the interstimulus interval, resulting in underestimation of the frequencies of superimposed mepps. Mepp amplitude was averaged from 20 to 60 mepps measured in the resting fiber and corrected using Eq. 4.

Quantal content was calculated by dividing the corrected epp amplitude by the corrected mepp amplitude. Quantal contents (Figs. 5 and 6) were plotted using both geometric and arithmetic averaging but since both analyses yielded similar patterns, only arithmetic averages are shown.

### Statistical analysis

Average values are reported as mean ± SE. Statistical significance was assessed using Student’s t-test, ANOVA, and Student-Newman-Keuls (SNK) or Tukey’s multiple comparison test using Instat/Prism software. Data were plotted using GraphPad Prism version 4.10 for Windows software (GraphPad Software, San Diego CA).

### Reagents

Fluorescent indicator dyes were purchased from Molecular Probes (Eugene, OR). Other reagents were from Sigma.

### RESULTS

**Mitochondria contribute to regulating stimulation-induced increases in cytosolic [Ca\(^{2+}\)] in low bath [Ca\(^{2+}\)]**

Figure 1A shows that the increase in rhod-2F fluorescence produced by 50-Hz stimulation in physiological saline was blocked by \( \Psi_m \) depolarization with CCCP, verifying the mitochondrial localization of AM-loaded rhod dyes. Figure 1B compares rhod-2 \( \Delta F/F \) responses in 2 and 0.5 mM Ca\(^{2+}\). In physiological [Ca\(^{2+}\)], mitochondrial [Ca\(^{2+}\)] rose above resting levels within 50–100 stimuli, and by 250–300 stimuli reached a plateau level that was maintained for the duration of the train, followed by a slow posttrain decay. Decreasing [Ca\(^{2+}\)] to 0.5 mM resulted in a slower rate of rise in mitochondrial [Ca\(^{2+}\)], though the final plateau value was unchanged. This plateau is not due to dye saturation because similar plateaus are measured in mitochondria loaded with lower-affinity dyes (David et al. 2003). David (1999) estimated that at this plateau level the free [Ca\(^{2+}\)] in the mitochondrial matrix is ~1 μM.

Figure 2 demonstrates the effects of bath [Ca\(^{2+}\)] and \( \Psi_m \) depolarization on stimulation-induced increases in cytosolic [Ca\(^{2+}\)], calculated from fluorescence changes of ionophoretically-injected OG-5N, as described in METHODS. Figure 2A shows that cytosolic [Ca\(^{2+}\)] increased rapidly within the first 1–2 s after the onset of stimulation, followed by a slower rate of rise for the duration of the train as reported by David et al. (1998). When stimulation ceased, cytosolic [Ca\(^{2+}\)] showed a rapid initial decay, followed by a slower decay to baseline. By the end of the 50-Hz, 10-s train, the increase in cytosolic [Ca\(^{2+}\)] above the assumed resting level of 0.1 μM was 0.65 ± 0.02 μM in 2 mM Ca\(^{2+}\), significantly greater than that in 0.5 mM Ca\(^{2+}\) (0.48 ± 0.02 μM, \( P < 0.01 \)). Stimulation after \( \Psi_m \) depolarization with antimycin A1 resulted in larger increases in cytosolic [Ca\(^{2+}\)] in both 2 and 0.5 mM Ca\(^{2+}\) with the magnitude of the response increasing over time (Fig. 2, B and C).

**FIG. 2.** \( \Psi_m \) depolarization increases peak cytosolic [Ca\(^{2+}\)] during 50-Hz stimulation in both physiological and low bath [Ca\(^{2+}\)]. A: increasing [Ca\(^{2+}\)] from 0.5 to 2 mM increases the cytosolic [Ca\(^{2+}\)] response. B: responses in 2 mM Ca\(^{2+}\) before (Con, ⋄) and 24 min (○) and 31 min (●) after \( \Psi_m \) depolarization with antimycin A1 (2 μM) in oligomycin (oligo, 5 μg/ml). C: responses in 0.5 mM Ca\(^{2+}\) before (Con, ⋄) and after \( \Psi_m \) depolarization (45 min, ▲; 53 min, ○; 61 min, ●). After \( \Psi_m \) depolarization exceeding 70 min, the peak of the [Ca\(^{2+}\)] response began to decline but remained above control levels (not shown). David (1999) showed that oligomycin alone has no effect on stimulation-induced [Ca\(^{2+}\)] responses. Cytosolic [Ca\(^{2+}\)] was calculated from changes in fluorescence of ionophoretically-injected OG-5N, assuming a resting level of 0.1 μM (see METHODS). All records are from a single terminal. Values in control solutions (no drugs) are the average of six to seven stimulus trains. Note the change in the [Ca\(^{2+}\)] scale among A–C.
Stimulation-induced cytosolic \([Ca^{2+}]i\) responses increased more rapidly and reached higher peak values in 2 than in 0.5 mM Ca\(^{2+}\). The measurements of vesicular and quantal release during \(\Psi_m\) depolarization reported below were made between 20 and 120 min after \(\Psi_m\) depolarization and thus would have been accompanied by increased cytosolic \([Ca^{2+}]i\) responses like those in Fig. 2, B and C.

The mitochondrial and cytosolic \(\Delta F/\Delta F_0\) responses in Figs. 1 and 2 demonstrate that mitochondria contribute to handling the Ca\(^{2+}\) loads associated with repetitive stimulation in low, as well as in physiological, bath \([Ca^{2+}]i\). In these terminals, mitochondria contribute more to Ca\(^{2+}\) sequestration than endoplasmic reticulum (ER), because cyclopiazonic acid, which blocks the ER Ca-ATPase, has no significant effect on cytosolic or mitochondrial \(\Delta F/\Delta F_0\) responses to 50-Hz stimulation (David 1999).

**Total vesicular release during 50-Hz stimulation increases after \(\Psi_m\) depolarization**

Figure 3 shows the effect of \(\Psi_m\) depolarization on total vesicular release (phasic plus asynchronous) during 50-Hz stimulation. The styryl dye FM2-10 was loaded into synaptic vesicles in 2 mM Ca\(^{2+}\), and preparations were then stimulated for 50 s (2,500 stimuli) in the indicated solutions. Destaining was measured and converted into % dye loss, as described in METHODS. In 2 mM Ca\(^{2+}\) (Fig. 3A) stimulation produced a 42 ± 4% dye loss in both control solution and oligomycin alone. After \(\Psi_m\) depolarization, total dye loss rose to 55 ± 4% by the end of the train, reflecting a significant increase in total vesicular release (P < 0.01, Tukey’s multiple comparison test). This increase in release was also significant after 10 s (500 stimuli), as used in Figs. 2 and 4-10; 19 ± 3% in CCCP vs. 11 ± 2% in control, P < 0.05).

This effect of \(\Psi_m\) depolarization on vesicular release was also seen in 0.5 mM Ca\(^{2+}\). Figure 3B shows that stimulation under control conditions produced a 16 ± 3% dye loss, ~40% of that measured in 2 mM Ca\(^{2+}\). Dye loss was similar in oligomycin alone but after \(\Psi_m\) depolarization, the percent dye loss increased to 31 ± 4%.

\(\Psi_m\) depolarization accelerates epp depression in 2 mM \([Ca^{2+}]i\), but transiently increases epp enhancement in 0.5 mM Ca\(^{2+}\).

Electrophysiological recordings were made to determine whether the \(\Psi_m\) depolarization-induced increase in vesicular release during 50-Hz stimulation was due mainly to phasic or asynchronous quantal release. Figure 4 shows representative traces of the first and last five epps evoked by a train of 500 stimuli. In 2 mM Ca\(^{2+}\), epp amplitude depressed, and this depression was increased during \(\Psi_m\) depolarization (Fig. 4A). In contrast, in 0.5 mM Ca\(^{2+}\), epp amplitudes increased both before and after \(\Psi_m\) depolarization (Fig. 4B). In both cases, there was evidence for increased asynchronous release at the end of the train during \(\Psi_m\) depolarization (see following text).

Figure 5 plots the averaged time course of epp depression during 50-Hz stimulation in 2 mM Ca\(^{2+}\). Epp amplitudes were

**FIG. 4. Effects of \(\Psi_m\) depolarization on phasic release during 50-Hz stimulation in 2 mM (A) and 0.5 mM (B) Ca\(^{2+}\). Representative epps recorded at the beginning (left) and end (right) of a 500 impulse 50-Hz train under control conditions (top) and in the presence of oligomycin and antimycin A1 (bottom). \(\Psi_m\) depolarization enhanced the depression of phasic release in 2 mM Ca\(^{2+}\) but did not depress phasic release in 0.5 mM Ca\(^{2+}\). Calibrations apply to both A and B. Stimulation artifacts were minimized for clarity.**
corrected for nonlinear summation, and quantal contents calculated as described in METHODS. Figure 5A plots averaged quantal contents measured in control, oligomycin alone, and oligomycin plus $\Psi_m$ depolarization. Final quantal contents in control and oligomycin alone were similar, but the final quantal content was significantly smaller during $\Psi_m$ depolarization.

Treatments that increase depression of phasic release during tetanic stimulation often increase the pretrain (baseline) quantal content. Table 1 indicates how these drug treatments affected phasic and asynchronous release from terminals at rest and during low-frequency stimulation (1 Hz). The low muscle resting membrane potentials were the result of having to depolarize the muscle fiber to minimize action potentials and subsequent contractions (see METHODS). Reducing bath [Ca$^{2+}$] from 2 to 0.5 mM reduced the average epp quantal content by $\sim$86%, from $\sim$50 to $\sim$7. However, for a given bath [Ca$^{2+}$], the applied drugs did not significantly alter resting membrane potentials or quantal content. Thus the increased depression during $\Psi_m$ depolarization in 2 mM Ca$^{2+}$ was not associated with a higher initial quantal content.

To examine the time course with which depression of phasic release developed, Fig. 5B plots quantal contents normalized to the pretrain quantal content, comparing control with oligomycin alone (left) and oligomycin alone with oligomycin plus $\Psi_m$ depolarization (right). In all conditions there was an initial increase in phasic release during the first 4 stimuli, followed by depression. At early times ($\sim$1 s) depression was greater in oligomycin alone than in control or $\Psi_m$-depolarized solutions, and in this normalized plot the final depression in oligomycin ($67 \pm 7\%$) exceeded that measured in control ($53 \pm 6\%$), though this difference failed to reach significance. By the end of the train the depression during $\Psi_m$ depolarization ($80 \pm 7\%$) was greater than that measured in control ($P < 0.05$). The semi-logarithmic plot in Fig. 5C shows that the time course of the late component of depression during $\Psi_m$ depolarization was faster than that in control solution or oligomycin alone.

FIG. 5. $\Psi_m$ depolarization accelerates the depression of phasic release during 50-Hz stimulation in 2 mM Ca$^{2+}$. A: averaged changes in quantal content ($m$) in control (left), oligomycin only (middle), and oligomycin plus $\Psi_m$ depolarization (right). ---, pretrain quantal content. B: pairwise comparisons of data in A after normalization to pretrain quantal content ($m_0$, calculated from 15 to 60 epps; average $m_0$ values are given in Table 1). Control (□), oligomycin only (○), and oligomycin plus $\Psi_m$ depolarization ( ● ). C: semi-logarithmic plot of the late component (5–10 s) of depression of normalized quantal content. Linear regressions yielded time constants and relative amplitudes (at $t = 0$) of (respectively) control: 36.2 s, 0.60; oligomycin: 31.1 s, 0.45; $\Psi_m$ depolarization plus oligomycin: 9.9 s, 0.50. (Con, control; O, oligomycin alone; O and $\Psi_m$, oligomycin plus $\Psi_m$ depolarization). D: earlier components of depression, after subtraction of slow component in C. Data in A–D are averages for 6–9 terminals in each condition.
Thus the increased depression of phasic release during $\Psi_m$ depolarization was not due solely to inhibition of mitochondrial ATP synthesis. Figure 5D shows the early components of depression, after subtraction of the late component. This plot suggests that during $\Psi_m$ depolarization a transient facilitatory process temporarily delayed the extra depression of phasic release that later ensued.

In contrast to the depression seen in 2 mM Ca$^{2+}$, 50-Hz stimulation in 0.5 mM Ca$^{2+}$ increased epp amplitude and quantal content under all conditions (Fig. 6A). Average normalized data in Fig. 6B show that in control solution phasic release increased during the first ~150 stimuli and then stabilized for the remainder of the train at ~3.4 times the pretrain value. In oligomycin alone, phasic release also increased and then plateaued at ~2.4 times the pretrain value. Stimulation after $\Psi_m$ depolarization enhanced phasic release by a maximum of ~4.5-fold, significantly greater than the enhancement in control and oligomycin-only solutions. After this peak at ~3–5 s, phasic release declined slightly.

Figures 5B and 6B plot quantal contents normalized to their pretrain values, the traditional way of plotting depression and facilitation. However, in both physiological and low [Ca$^{2+}$] conditions, the pretrain quantal contents in oligomycin alone tended to be higher than those measured in control solution, although this difference did not reach statistical significance (Table 1). This tendency toward higher initial quantal contents might have contributed to the finding that in oligomycin alone the normalized quantal contents showed greater depression than control values as stimulation progressed.

During 50-Hz stimulation average quantal contents in 0.5 and 2 mM Ca$^{2+}$ converge

Comparison of Figs. 5A and 6A demonstrates the surprising finding that, under five of the six experimental conditions examined, the final average quantal contents achieved during 50-Hz stimulation were similar. Figure 7 plots the time course with which these quantal contents converged. In both control solutions (Fig. 7A) and oligomycin alone (Fig. 7B) the initial quantal contents were large (50–57) in 2 mM Ca$^{2+}$ and small (7–8.5) in 0.5 mM Ca$^{2+}$, but during 50-Hz stimulation the quantal contents converged to a similar value (~20) within ~6 s. Toward the end of the 50-Hz train, epss in 0.5 mM Ca$^{2+}$ occasionally produced an action potential (not shown). Because for this study we selected partially depolarized muscle fibers in which the threshold for action potential generation was higher than normal, we suspect that in normally polarized muscle, the depolarization produced by 20 quanta would have been sufficient to elicit an action potential most of the time. After $\Psi_m$ depolarization (Fig. 7C) in 0.5 mM Ca$^{2+}$ quantal contents reached a similar value, but in 2 mM Ca$^{2+}$ quantal contents continued to depress throughout the course of stimulation.

Repetitive stimulation produces more asynchronous release after $\Psi_m$ depolarization

Figure 8 plots the average increase in asynchronous release measured during stimulus trains applied under control conditions, in oligomycin alone, and after $\Psi_m$ depolarization in 2

![Image](http://jn.physiology.org/)

**FIG. 6.** In 0.5 mM Ca$^{2+}$ $\Psi_m$ depolarization transiently enhances phasic release during 50-Hz stimulation. A: pairwise comparisons of changes in average quantal content ($m$) during stimulation in control (○), in oligomycin alone (△), and following $\Psi_m$ depolarization (●). B: data in A are replotted with quantal content normalized to pretrain values ($m_0$; Table 1 gives average $m_0$ values). At midtrain the average $m/m_0$ values after $\Psi_m$ depolarization were significantly different from control ($P < 0.05$) and from oligomycin alone ($P < 0.001$, stimuli 200–250 between 4 and 5 s, 3.35 ± 0.27 in control, 2.43 ± 0.14 oligomycin alone, 4.54 ± 0.65 $\Psi_m$ depolarization) Values at the end of the train were not significantly different. Curves were smoothed with a bin size of 9, and values from 6 to 9 terminals were averaged for each condition.

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To make it difficult to detect all superimposed mepps. Posttrain measurements often revealed higher mepp frequencies not discernible during the train. An example is shown in Fig. 9, where the measured asynchronous release rate increased from \( \sim 300 \) s\(^{-1}\) during tetanic stimulation to almost 600 s\(^{-1}\) immediately after stimulation. Third, the need to use partially depolarized muscle fibers (especially in 2 mM Ca\(^{2+}\), see METHODS) reduced mepp amplitudes, making them harder to detect. A decrease in vesicular acetylcholine content during repetitive stimulation could also contribute to an underestimation of asynchronous release (Naves and van der Kloot 2001). For these reasons, asynchronous release rates during trains must have been greater than the values measured here, with larger errors likely in 2 mM Ca\(^{2+}\) and at the higher mepp frequencies.

Histograms in Fig. 10 show average cumulated phasic and asynchronous quantal release during the stimulus trains. In 2 mM Ca\(^{2+}\), \( \Psi_m \) depolarization significantly decreased summed phasic release but increased summed asynchronous release by almost 20-fold. In 0.5 mM Ca\(^{2+}\), summed asynchronous release increased \( \approx 10 \)-fold during \( \Psi_m \) depolarization, but summed phasic release was not significantly different from control. In both 2 and 0.5 mM Ca\(^{2+}\), total release (phasic plus asynchronous) was not significantly different across treatments, although \( \Psi_m \) depolarization increased the percentage of release that was asynchronous. In contrast, the dye-destaining study of Fig. 3 indicated greater total vesicular release during \( \Psi_m \) depolarization. This discrepancy joins with the reasons noted in the preceding text to suggest that asynchronous release during \( \Psi_m \) depolarization was undercounted. Thus the data of Fig. 10 likely underestimate the degree to which release was desynchronized during 50-Hz stimulation when \( \Psi_m \) was depolarized.

More detailed comparisons between vesicular release estimated by dye-destaining and quantal release measured by electrophysiology were not possible because, in addition to the undercounting of asynchronous quanta, the duration of stimulation used for the electrophysiological experiments (10 s at 50 Hz) was short compared with the train durations usually used for dye-destaining experiments. Dye measurements over short intervals are complicated by the kinetics of destaining, which usually begins with a lag after the onset of stimulation. (In our experience, this lag was less for FM2-10 than for FM1-43, perhaps because FM2-10 is less hydrophobic than FM1-43.) We were constrained to use shorter durations of stimulation in the electrophysiological experiments because of the need to administer multiple stimulus trains in most experiments. Use of longer durations would have required intertrials intervals even longer than the 10 min used here.

**DISCUSSION**

Results of this study demonstrate that mitochondria are relevant for handling Ca\(^{2+}\) loads in low (0.5 mM) as well as in physiological 2 mM Ca\(^{2+}\) because under both conditions, [Ca\(^{2+}\)] in the mitochondrial matrix increased during 50-Hz stimulation (Fig. 1), and stimulation-induced elevations of cytosolic [Ca\(^{2+}\)] became greater when Ca\(^{2+}\) sequestration was inhibited by depolarizing \( \Psi_m \) (Fig. 2). Our finding that stimulation-induced increases in mitochondrial [Ca\(^{2+}\)] reached similar plateau values in both low and physiological [Ca\(^{2+}\)] agrees with previous work showing that the plateau amplitude does
not vary when stimulation frequency is varied from 20 to 100 Hz or when Ca\textsuperscript{2+} influx per action potential is increased or decreased (David 1999; David et al. 2003). David (1999) hypothesized that this constant plateau amplitude results from reversible formation of an osmotically inactive calcium complex within the matrix.

**Effects of inhibiting mitochondrial ATP synthesis without \( \Psi_m \) depolarization**

Inhibition of mitochondrial ATP synthesis with oligomycin (which does not depolarize \( \Psi_m \)) had relatively little effect on stimulation-induced changes in total vesicular release (Fig. 3) or on phasic and asynchronous quantal release (Fig. 10). This finding is consistent with the previous demonstration that oligomycin had no significant effect on stimulation-induced increases in cytosolic [Ca\textsuperscript{2+}] in this preparation (David 1999). Oligomycin did appear to reduce phasic release during 50-Hz stimulation in normalized records in both physiological and low-bath [Ca\textsuperscript{2+}] (Figs. 5B and 6B), but this effect may have been related to the slightly higher initial quantal contents measured in oligomycin. In nonnormalized measurements, it was clear that the brief oligomycin exposures used here had little or no effect on the terminals’ ability to sustain phasic release during maintained stimulation. Because some steps in vesicular trafficking require ATP (Heidelberger et al. 2002; Klenchin and Martin 2000; Ohyama et al. 2002), our findings suggest that oligomycin alone did not produce severe ATP depletion in these motor terminals. Perhaps ATP sufficient to sustain release diffused into the terminal from the axon, which is partially protected from the effects of bath-applied drugs by myelin and the perineural sheath. Motor terminals and axons can also make ATP by glycolysis. Peripheral motor axons have been shown to be more resistant to ischemia than central axons, perhaps due to a greater capacity for anaerobic metabolism (reviewed by Stys et al. 1995). The glycolytic capacity of motor axons/terminals probably helps to sustain release as the partial pressure of O\textsubscript{2} falls to low levels within tetanically stimulated muscles (Koch 2002; Richardson et al. 1999; Wagner 2001).

**Desynchronization of release during \( \Psi_m \) depolarization**

For the \( \leq 2 \) h exposure times used here, \( \Psi_m \) depolarization had no significant effect on the baseline mepp frequency or on

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**FIG. 8.** \( \Psi_m \) depolarization increases asynchronous release during 50-Hz stimulation in both 2 mM (A) and 0.5 mM (B) Ca\textsuperscript{2+}. Changes in mepp frequency are plotted in control solutions, after addition of oligomycin, and during \( \Psi_m \) depolarization. Mepps were detected during the latter half of each interstimulus interval using the Synaptosoft program. Counts from each terminal were first smoothed (bin = 5) and then averaged (5–8 terminals for each condition). Counts exceeding 100 s\textsuperscript{–1} are underestimates (see text).

**FIG. 9.** Mepp frequencies measured for a representative terminal during and after 50-Hz stimulation with \( \Psi_m \) depolarization in 0.5 mM Ca\textsuperscript{2+}. The dramatic increase in asynchronous release measured immediately after stopping stimulation suggests that mepp frequency was underestimated during stimulation. The rapid posttrain decline in asynchronous release was observed in all terminals examined.

**FIG. 10.** Cumulative phasic and asynchronous release during a 500-stimulus train for 2 and 0.5 mM bath Ca\textsuperscript{2+} in control, in oligomycin alone, and after \( \Psi_m \) depolarization. In 2 mM Ca\textsuperscript{2+} cumulative phasic release was significantly reduced after \( \Psi_m \) depolarization (*, \( P < 0.01 \), SNK test). Asynchronous release during \( \Psi_m \) depolarization was significantly different from control for both 2 and 0.5 mM Ca\textsuperscript{2+} (*, \( P < 0.01 \)). Values are the average of 6–9 terminals in each condition.
depolarization increased the rate of asynchronous release during stimulus trains in both low and physiological bath [Ca\(^{2+}\)]. One likely reason is the greater elevation of cytosolic [Ca\(^{2+}\)] recorded in response to stimulation during \(\Psi_m\) depolarization. Under control conditions, mitochondrial Ca\(^{2+}\) sequestration restricted the elevation of spatially averaged cytosolic [Ca\(^{2+}\)] to \(\leq 1\) \(\mu M\), but much higher levels were attained when this sequestration was inhibited by \(\Psi_m\) depolarization (Fig. 2, B and C) (David 1999; David et al. 1998). The marked increase in asynchronous release measured under these conditions is consistent with reports that motor and some other presynaptic terminals and secretory cells contain release mechanisms with \(K_d < 10\) \(\mu M\) (Angleson and Betz 2001; Augustine and Neher 1992; Bittner and Holz 1992; Bollmann et al. 2000; Ravin et al. 1997; Rieke and Schwartz 1996; Schneggenburger and Neher 2000). As expected, the stimulation-induced elevations of both cytosolic [Ca\(^{2+}\)] and asynchronous release during \(\Psi_m\) depolarization were greater in 2 mM than in 0.5 mM Ca\(^{2+}\), but our data were not sufficient to permit quantification of the relationship between cytosolic [Ca\(^{2+}\)] and asynchronous release. One reason was that during \(\Psi_m\) depolarization the stimulation-induced elevations in cytosolic [Ca\(^{2+}\)] changed over time (Fig. 2), and we have not yet succeeded in measuring cytosolic [Ca\(^{2+}\)] and release simultaneously from the same terminal. Another reason was that asynchronous release rates evoked by stimulation during \(\Psi_m\) depolarization exceeded the ability for accurate measurement by the counting method as also found by David and Barrett (2003) in mouse motor terminals.

\(\Psi_m\) depolarization increased phasic release during 50-Hz stimulation in 0.5 mM Ca\(^{2+}\), but this increase was transient, because as stimulation continued, release returned to the levels measured in non-\(\Psi_m\) depolarized preparations (Fig. 6B). Effects of \(\Psi_m\) depolarization during stimulation in 2 mM Ca\(^{2+}\) were qualitatively similar, but in this case, the early facilitation was smaller (Fig. 5D) and the ensuing depression of phasic release was more profound (Fig. 5C). The marked difference between the effects of oligomycin alone and \(\Psi_m\) depolarization indicates that the effects of \(\Psi_m\) depolarization on phasic release were not due solely to inhibition of mitochondrial ATP synthesis. Rather, it appears that these effects were due, directly or indirectly, to the greater stimulation-induced elevations of cytosolic [Ca\(^{2+}\)] recorded during \(\Psi_m\) depolarization or to the combination of elevated cytosolic [Ca\(^{2+}\)] and reduced ATP synthesis. The modest elevations of cytosolic [Ca\(^{2+}\)] at the onset of stimulation during \(\Psi_m\) depolarization would be expected to facilitate phasic release (in accord with “residual Ca\(^{2+}\)” models, reviewed in Zucker and Regehr 2002), but greater or longer-lasting [Ca\(^{2+}\)] elevations have been reported to depress phasic release (Adams et al. 1985; Augustine and Neher 1992; Hsu et al. 1996). This depression was likely not due to a decrease in the sensitivity of postsynaptic receptors, because mepps were readily detected throughout and after the 50-Hz tetanus (Fig. 9). Nor was the depression likely due to failure of action potential conduction because epps continued to be recorded throughout the stimulus train. Reduced phasic release when cytosolic [Ca\(^{2+}\)] becomes excessively elevated might be caused by direct effects, e.g., “adaptation” of the release mechanism (Hsu et al. 1996) or inhibition of N-type Ca\(^{2+}\) channel activity (Shirokov 1999) or by indirect effects associated with the increased asynchronous release. During 50-Hz stimulation in both 2 and 0.5 mM Ca\(^{2+}\), the ratio of asynchronous release to phasic release increased, i.e., release became desynchronized (see also David and Barrett 2003; Kirischuk and Grantyn 2003). Excess asynchronous release might inhibit phasic release via activation of inhibitory autoreceptors on the motor terminal (reviewed by Wu and Saggau 1997) or via competition if both types of release share a vesicle pool (as suggested by Hagler and Gода 2001) and/or if release of one vesicle transiently depresses the likelihood of further release from the same active zone (Stevens and Tsujimoto 1995).

Convergence of phasic release in physiological and low bath [Ca\(^{2+}\)] during repetitive stimulation

A striking feature of our data was that the phasic release measured in 2 and 0.5 mM Ca\(^{2+}\) converged to a common quantal content (~20) during repetitive stimulation (Fig. 7). This quantal content would have been adequate to sustain neuromuscular transmission in muscle fibers with a normal resting potential and thus may be just sufficient to sustain release during prolonged tetanic stimulation. This ability to attain suprathreshold release rates even in low-bath [Ca\(^{2+}\)] would preserve neuromuscular transmission if [Ca\(^{2+}\)] in the synaptic cleft falls during repetitive stimulation (a possibility discussed in Borst and Sakmann 1999; Ginsburg and Ramahmoff 1983; Stanley 2000). The convergence of epp quantal content measured here is reminiscent of the convergence of total release (phasic plus asynchronous) noted by Hagler and Gода (2001) in hippocampal autaptic synapses in vitro during 20-Hz stimulation in varying bath [Ca\(^{2+}\)].

This convergence of quantal content might not seem surprising given the abundant evidence that during sustained repetitive stimulation, phasic release in physiological [Ca\(^{2+}\)] depresses whereas phasic release in lower [Ca\(^{2+}\)] potentiates. But the mechanism by which the terminal achieves this convergence may shed light on synaptic control mechanisms. A full examination of such mechanisms is beyond the scope of this study, but the data of Fig. 2A suggest that one mechanism contributing to the convergence involves control of the stimulation-induced elevation of cytosolic [Ca\(^{2+}\)]. If this elevation was proportional to bath [Ca\(^{2+}\)], one would have expected a fourfold greater elevation in 2 than in 0.5 mM [Ca\(^{2+}\)], but the measured elevations indicated a difference of only ~1.3-fold near the end of the stimulus train. This similarity of the elevations of cytosolic [Ca\(^{2+}\)] in 0.5 and 2 mM Ca\(^{2+}\) suggests that during repetitive stimulation Ca\(^{2+}\) influx becomes a less-than-linear function of bath [Ca\(^{2+}\)] and/or that intraterminal Ca\(^{2+}\) sequestration/buffering/extrusion mechanisms become greater-than-linear functions of cytosolic [Ca\(^{2+}\)]. Mechanisms that might make Ca\(^{2+}\) influx a less-than-linear function of bath [Ca\(^{2+}\)] include inhibition of N-type Ca\(^{2+}\) channels by elevated cytosolic [Ca\(^{2+}\)] or by inhibitory autoreceptors (references cited in the preceding text) or the Ca\(^{2+}\)-activated K\(^+\) conductance in these terminals (Morita and Barrett 1990), which shortens the duration of the action potential as bath [Ca\(^{2+}\)] increases. An intraterminal Ca\(^{2+}\) handling mechanism with a greater-than-linear dependence on cytosolic [Ca\(^{2+}\)] is the...
opening of the mitochondrial uniporter (reviewed by Gunter and Pfeiffer 1990).

Interestingly, the quantal content at the end of a 500-stimulus, 50-Hz train was similar not only over a range of bath [Ca$^{2+}$], but also when mitochondrial ATP synthesis was inhibited by oligomycin. Phasic release was even well sustained during brief (<2 h) $\Psi_{m}$ depolarization in low bath [Ca$^{2+}$]. In all these conditions, the stimulation-induced increase in cytosolic [Ca$^{2+}$] remained modest (<5 µM). But phasic release was not well sustained during $\Psi_{m}$ depolarization in physiological [Ca$^{2+}$], where the stimulation-induced elevation of cytosolic [Ca$^{2+}$] rapidly reached higher levels. These findings suggest that mitochondrial Ca$^{2+}$ uptake is most critical at higher rates of Ca$^{2+}$ influx and that limiting the increase in spatially averaged cytosolic [Ca$^{2+}$] during repetitive stimulation is important for sustaining phasic release and thereby neuromuscular transmission.

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