Augmentation Controls the Fast Rebound From Depression at Excitatory Hippocampal Synapses

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García-Perez E, Wesseling JF. Augmentation controls the fast rebound from depression at excitatory hippocampal synapses. J Neurophysiol 99: 1770–1786, 2008. First published January 16, 2008; doi:10.1152/jn.01348.2007. Short-term plasticity occurs at most central chemical synapses and includes both positive and negative components, but the principles governing interaction between components are largely unknown. The residual Ca$_{2+}$ that persists in presynaptic terminals for several seconds after repetitive use is known to enhance neurotransmitter release under artificial, low probability of release conditions where depression is absent; this is termed augmentation. However, the full impact of augmentation under standard conditions at synapses where depression dominates is not known because of possibly complicated convolution with a variety of potential depression mechanisms. This report shows that residual Ca$_{2+}$ continues to have a large enhancing impact on release at excitatory hippocampal synapses recovering from depression, including when only recently recruited vesicles are available for release. No evidence was found for gradual vesicle priming or for fast refilling of a highly releasable recruited vesicle pool (RRP). And decay of enhancement matched the clearance of residual Ca$_{2+}$, thus matching the behavior of augmentation when studied in isolation. Because of incomplete RRP replenishment, synaptic strength was not typically increased above baseline when residual Ca$_{2+}$ levels were highest. Instead residual Ca$_{2+}$ caused single pulse release probability to rebound quickly from depression and then depress quickly during subsequent bursts of activity. Together, these observations can help resolve discrepancies in recent timing estimates of recovery from depression. Additionally, in contrast to results obtained under reduced release conditions, augmentation could be driven to a maximal level, including paired-pulse facilitation and other mechanisms that increase release efficiency.

INTRODUCTION

Recently there has been substantial interest in the mechanisms that underlie short-term plasticity at central synapses because it has become clear that this phenomenon plays an important role in neural circuit function (Abbott et al. 1997; Chung et al. 2002; Cook et al. 2003; Markram et al. 1998; Nadim et al. 1999; Silberberg et al. 2004). Almost all synapses exhibit some form of short-term plasticity with some types of synapses mostly strengthening when used repetitively and others mostly weakening—i.e., short-term enhancement and short-term depression. However, the same synapses can exhibit both forms; most central synapses eventually depress when used extensively, but many of these first strengthen transiently at the beginning of a repetitive train (Zucker and Regehr 2002).

Short-term enhancement has been kinetically subdivided into several elemental processes, and at least some of these are mechanistically distinct from factors involved in depression. That is, some types of short-term depression are thought to be caused by depletion of a small, readily releasable pool (RRP) of primed vesicles in presynaptic terminals that are directly available to be triggered for release by action potentials (Zucker and Regehr 2002). In contrast, most elements of enhancement are driven by low levels of residual Ca$_{2+}$ that accumulate presynaptically during action potential firing, and, at least one element, previously termed augmentation, is known to arise from the potentiated efficacy of the release machinery itself; i.e., augmented synapses are stronger because the probability of release per available vesicle within the RRP is increased and not because of changes in the number of release-ready vesicles (Magleby and Zengel 1976b; Stevens and Wesseling 1999a; see also Rosenmund et al. 2002). Thus residual Ca$_{2+}$ may continue to play a role in determining synaptic strength even after synapses have become depressed due to RRP depletion.

However, enhancement has typically been studied in isolation from depression with manipulations that limit coincident depletion of the RRP (Feng 1941; Hubbard 1963; Liley and North 1953; Magleby and Zengel 1976a), and the functional independence of enhancement from mechanisms of depression has never been tested thoroughly. Indeed it has been suggested that newly recruited readily releasable vesicles are primed for release gradually, at least at Calyx of Held synapses, which causes a reduction in P$_{VES}$ that contributes to depression (Wu and Borst 1999), and this additional mechanism might block, dampen, or simply subtract from, the enhancement of P$_{VES}$ that would otherwise be expected in the presence of residual Ca$_{2+}$. While one report has shown that residual Ca$_{2+}$ can enhance release parameters in the presence of masking depression at neuromuscular junctions (Kalkstein and Magleby 2004), two studies of excitatory hippocampal synapses (Brager et al. 2003; Klyachko and Stevens 2006) provided estimates of the timing of decay of enhancement under standard conditions that were up to fivefold slower than under low probability of release conditions (Fisher et al. 1997; Stevens and Wesseling 1999a) and also substantially slower than the clearance of residual Ca$_{2+}$ (Brager et al. 2003). As the mismatch between the decay of enhancement and residual Ca$_{2+}$ clearance would be a predicted consequence of convolving gradual vesicle priming with enhancement, these findings together suggest that gradual vesicle priming may play a substantial role in determining the timing of recovery from synaptic depression at a range of synapse types, from large Calyces of Held to small excitatory...
synapses of the hippocampus. An open question, then, is: what sort of impact do classically defined elements of short-term enhancement, such as augmentation, have at times when synapses are still recovering after the induction of short-term depression mechanisms that possibly include both RRP depletion and gradual/incomplete priming of the newly recruited vesicles that have become available?

We report here that residual Ca\(^{2+}\)–dependent enhancement of release plays a substantial role during the first several seconds of recovery from depression at hippocampal synapses and, surprisingly, that the impact of gradual vesicle priming appears to be small or nonexistent during recovery intervals lasting ≥1 s. The impact of enhancement during recovery intervals 1) can be large, as much as 4-fold in this study, 2) persists for several seconds, 3) tracks residual Ca\(^{2+}\), and 4) is likely due to the same residual Ca\(^{2+}\)–dependent mechanism that underlies augmentation as defined under low probability of release conditions; the enhancement of \(P_{\text{ves}}\) for newly recruited vesicles exhibits key kinetic and residual Ca\(^{2+}\) dependences that fulfill the original criteria for augmentation (Delaney and Tank 1994; Magleby and Zengel 1976a; Zengel and Magleby 1982). These conclusions differ in some respects with previous reports, but differences can be attributed to differences in methods of analysis as will be further described in the DISCUSSION.

Thus our data show that low-level residual Ca\(^{2+}\) that is cleared slowly from presynaptic terminals following repetitive use seems to have a general, enhancing effect on neurotransmitter release that is mechanistically independent of the state of RRP depletion and replenishment, at least at excitatory synapses of the hippocampus. Phenomenologically, however, most of the enhancement at synapses that are recovering from depression is not observed as an increase in synaptic strength above baseline values because of masking by concurrent RRP depletion. Under standard conditions, residual Ca\(^{2+}\) instead causes a dynamic acceleration of the timing of the release events evoked during bursts of stimulation, which is manifest 1) as a quick rebound from depression in the probability of release (after single pulses) that outpaces the time course of RRP recovery, but also 2) as the faster onset of depression during a 2nd burst initiated within a few seconds of the 1st one, as expected because of the faster disbursement of an only partially full RRP. These observations have important implications for theoretical analyses of how dynamic synaptic behavior is used to support biological computation and also key technical implications concerning experiments designed for probing the cell biological mechanisms underlying short-term plasticity.

**METHODS**

Animal sacrifice was performed by decapitation and was conducted in accord with Spanish royal decree 1201/2005. All synaptic responses were measured from patch clamped neurons held in whole cell voltage-clamp mode with typical pipette resistances of 3–5 MΩ.

**Slice preparation**

Transverse slices (400 μm thick) were prepared from the hippocampi of 2- to 3-wk-old mice as described in detail previously (Wesseling and Lo 2002). Area CA3 was removed for all experiments. The extracellular recording solution contained (in mM) 120 NaCl, 1.25 NaH\(_2\)PO\(_4\), 26 NaHCO\(_3\), 10 glucose, 3.5 KCl, picrotoxin (50 μM), and 5-2-amino-5-phosphonovaleric acid (d–) APV (50 μM) and was continuously bubbled with a mixture of 95% O\(_2\)-5% CO\(_2\) starting ≥20 min prior to addition of CaCl\(_2\) (usually 2.6 mM, except as indicated) and MgCl\(_2\) (usually 1.3 mM). Recording pipettes were filled with a solution containing (in mM) 130 Cs-glucuronate, 5 CsCl, 5 NaCl, 2 MgCl\(_2\), 2 MgATP, 0.2 LiGTP, 1 EGTA, 0.2 CaCl\(_2\), and 10 HEPES, adjusted to a pH of 7.2, and an osmolarity of 290 mosM. Excitatory postsynaptic currents were evoked in CA1 pyramidal neurons with constant current pulses of <250 μA (almost always <75 μA) for ≤100 ms via a monopolar silver/silver chloride electrode inserted into a glass pipette (tip diameter between 20 and 40 μm), filled with recording solution, and placed toward the CA3 end of the stratum radiatum.

**Cell culture preparation**

Isolated neurons were grown in cell culture essentially as described previously (Stevens and Wesseling 1998). Glass cover slips were coated with agarose (0.15%, type IIA) and allowed to dry before application of a particulate mist of substrate solution (1 mg/ml rat tail collagen, and 0.05 mg/ml poly-D-lysine) with an airbrush. Tissue from newborn mouse hippocampi was dissociated with papain (15 units/ml, Worthington) dissolved in minimum essential media (MEM, Gibco, no glutamine) for 30 min at 37°C and then washed with culture media containing 10% horse serum in MEM, penicillin (100 units/ml), streptomycin (100 μg/ml), sodium pyruvate (1 mM), and N-2 supplement (1×) from Gibco. Tissue was then triturated mechanically with a pipette. Cells were plated at ∼3 × 10^4 cells (0.5 ml) per well in 24-well cell culture plates and were grown for 10 or 11 days in a 5% CO\(_2\) incubator (37°C) before use.

For cell culture experiments, the extracellular recording solution contained (in mM) 132 NaCl, 2 KCl, 10 glucose, 15 sorbitol, 10 HEPES, d–APV (50 μM), and CaCl\(_2\) and MgCl\(_2\) as indicated. Recording pipettes were filled with (in mM) 140 K-glucuronate, 9 NaCl, 1 MgCl\(_2\), 2 MgATP, 0.2 LiGTP, 1 EGTA, 0.2 CaCl\(_2\), and 10 HEPES. Both solutions were adjusted to a pH of 7.2, and an osmolarity of 295–305 mosM. Uncontrolled action potentials were evoked presynaptically by transiently depolarizing cell bodies as described in Bekkers and Stevens (1991). Osmotic shocks were induced with hyperosmotic saline containing 1.6 mM CaCl\(_2\), 1.6 mM MgCl\(_2\), and 500 mM sucrose, which was applied by picospritzing from a glass pipette with a tip diameter of between 2 and 3 μm and was cleared rapidly with a vacuum pipette with a tip diameter of 10–50 μm.

**Experimental design**

In general, it was often possible to repeat several trials of each experiment on individual preparations. To allow the synapses to recover completely between trials, ≥4 min were allowed for rest before stimulation was initiated for each trial in the slice preparation (3 min for the near-minimal stimulation experiments), and 1 min in the culture preparation. For the experiments with a single experimental variable, the experimental and control trials were alternated. For time courses, the order of trials was shuffled. Access resistance was monitored with –10-mV voltage steps before and after each trial, and data were only accepted if no change was detected during each trial, and also between trials for Figs. 2C and 6, A and B, and Supplementary Fig. S1.1 For Fig. 2C, experimental trials were interleaved with matched control trials where the stimulation frequency was maintained at 20 Hz throughout, and were repeated at least three times before adding DNQX; identical trials were averaged before further analysis.
**Analysis of electrophysiology**

Except as noted, synaptic responses were measured as the current integral. Rise times and peak current measurements gave similar results for the slice recordings (Wesseling and Lo 2002) but not always for the recordings obtained in cell culture because of activity dependent changes in asynchronous release in cell culture that do not seem to play a role in the slice preparation (Hagler and Goda 2001; Otsu et al. 2004).

**RRP recovery time courses**

RRP recovery values were estimated from paired trains of responses (e.g., diagram above Fig. 2A) in three steps. 1) An initial value was obtained by dividing the sum of the sizes of the 1st 60 responses during the 2nd train by the sum of the 1st 60 responses during the 1st train (in many cases the 1st and 2nd trains were longer than 60 pulses, but only the 1st 60 responses were needed for this analysis). The initial value is thought to be linearly related to the amount of RRP recovery occurring during the inter train interval, but would not be expected to equal 0 at times when RRP was empty, because of continued recruitment and release of new vesicles during stimulation (Wesseling and Lo 2002). Therefore, 2) the initial value for each recovery interval was adjusted by subtracting the initial value obtained from trials conducted with no rest interval between trains. 3) The adjusted value was subsequently normalized to vary from 0 to 1 by dividing by the range (i.e., 1 minus the initial value obtained for the no rest interval trials).

**Near-minimal stimulation**

Near-minimal stimulator settings were determined during low-frequency stimulation as the intensity needed to elicit successful synaptic transmission less than half of the time. To limit the likelihood of failures arising from axonal threshold fluctuations, stimulus settings were used only when it was possible to both increase and decrease the synaptic transmission less than half of the time. To limit the likelihood of failures arising from axonal threshold fluctuations, stimulus settings were used only when it was possible to both increase and decrease the intensity by ≥2.5% without noticeably changing the probability of release (Allen and Stevens 1994; Raastad et al. 1992; see Fig. 1B of Wesseling and Lo 2002 for control experiments showing that axon firing threshold is stable with repetitive use).

**Ca2+ imaging**

Ca2+ indicator dye was prepared and loaded into slices essentially as described by (Wu and Saggau 1994). Dye (50 µg) was dissolved in 5 µl DMSO containing 20% Pluronic F-127 (Sigma), vortexed, diluted with 45 µl extracellular solution, which sometimes contained Alexa Fluor 488 hydrazide (100 µM, used for monitoring the loading procedure), and filtered at 0.22 µm. All dyes were from Molecular Probes. Dye solution was loaded into the tip of a glass pipette (tip diameter = 2.5–3 µM) and pressure injected into the s. radiatum with a picospritzer for 5–15 min; care was taken to use low injection pressures to avoid obvious damage to the slice. The stimulation pipette was placed near the injection site within the dye spot (radius: ~75 µM), which was clearly visible (dark) with halogen light illumination immediately after injection (~5 objective). Subsequently, a ×40 objective was aligned above a region of the s. radiatum ~250 µM from the injection site and ≥100 µM from the edge of the original dye spot.

Optical recording was initiated after waiting ≥1 h for dye transport down the axons. Fluorescence changes were monitored at 5 or 1 Hz (indicated in figure legends) by switching on and off one of two discrete light-emitting diodes (LEDs; exposure length of 30 or 100 ms, LEDtronics, either pure blue BP280CWPB3K-3.6V-050T, or ultraviolet BP2000CUV750-250). Excitation and emission light was filtered with standard filter sets purchased from Omega Optical (either XF100-2 for Fluor or XF04-2 equipped with a 380 band-pass excitation filter). After passing through the objective and tube lens within the microscope frame (Olympus BX 51), emission light passed through a digital iris diaphragm, which was used to eliminate light from areas outside of the s. radiatum (imaged area diameter was ~150 µM); a plano-convex singlet lens (F = 50 mm) was then used to project this spatially restricted image onto a silicon avalanche photodiode (Perkin Elmer, C30902E).

At least three (Fluo 4) or 15 (Furaptra) trials were performed for each experiment and averaged before further analysis. Linear rundown in fluorescence signal due to photo-bleaching was nominally subtracted before analysis, but various combinations of neutral density filters were placed in the excitation path to ensure that this was negligible in all cases (see insets of Figs. 2D, 4C, and 5C). In all experiments, a permanent, activity dependent change of ~0.5% was detected in steady-state fluorescence levels—possibly due to dye extrusion—but this was only readily noticeable in the Furaptra experiments where stimulation produced an extremely weak signal (see Fig. 2D). Additional experimental controls (not shown) showed that the amplitude but not the decay time course of the fluorescence signal depended on stimulation strength; i.e., this was expected because stronger stimuli elicit action potentials in more axons. No attempt was made to take advantage of the ratiometric properties of Furaptra for determining Ca2+ concentrations because this would require spatial heterogeneity in Ca2+ levels, and this condition was not met because stimulation would activate only a minor fraction of dye loaded terminals.

**RESULTS**

Examples of the main phenomenon analyzed here are presented in Fig. 1, which shows that although synaptic strength appears to recover quickly from depression when tested with individual pulses, recovery to full functionality takes longer. That is, during paired trains of 40-Hz Schaffer collateral stimulation with each train consisting of ≥60 pulses, the response to the first pulse of the second train, initiated after a 2-s rest interval, was nearly as large as the response to the first pulse of the first train (Fig. 1A1i, inset). In contrast, the aggregate response during the entire second train was substantially smaller than the aggregate response during the first train (Fig. 1A1i) because of the more rapid induction of depression during the second train; it took 43 pulses during the first train to drive the synapses to within 10% of steady state but only 26 pulses during the second train (Fig. 1Ai).

Likewise in near-minimal stimulation experiments where quantal responses could be resolved because only one or a few afferents were activated, the probability of release after the first pulse of the second train recovered completely during the 2-s inter-train interval (Fig. 1Bi, 0.38 ± 0.15, compared with 0.36 ± 0.06, mean ± SE, 3 recordings, ≥10 trials per recording), while the total number of neurotransmission successes during the second train was significantly less than the total number during the first train (14.3 ± 1.0 vs. 25.6 ± 1.3, n = 47 trials). A χ² test confirmed that the probability of release in response to the first pulse of the second train recovered significantly more during the 2-s rest interval than did recovery of the number of successes during the entire second train (P < 0.05). The mean amplitudes of successes after the second to sixth pulses of the first train were larger than the mean amplitude at later times during the first train and during the entire second train, suggesting that the number of successes during the first train was likely an underestimate of the number of quanta released owing to the simultaneous release of multiple quanta (see Fig. 1Bii); correction for the underestimate...
was not necessary for the $\chi^2$ test, however, because the simultaneous release of multiple quanta was likely not a factor after the first pulse of the first train (see legend of Fig. 1Bii).

We interpret the incomplete recovery of the aggregate response, or total number of neurotransmission successes, during the second train as an indication that a RRP of presynaptic vesicles was emptied during the first train and only had enough time to recover partially during the 2-s rest interval. Evidence for this was published in an earlier report (Wesseling and Lo 2002), and includes key control experiments showing 1) that dynamic changes in synaptic strength are presynaptic in this preparation when long-term changes are prevented by blocking NMDA receptors, 2) that higher frequency stimulation, or higher extracellular Ca$^{2+}$ levels, dramatically alter the timing of release events during the trains, but the only changes seen in the total amount of release for trains consisting of at least 60 pulses are predicted second order effects owing to recruitment of new vesicles, and 3) that the rate of transmitter release is no longer sensitive to the frequency of stimulation after the 1st 60 pulses as long as stimulation frequency is maintained at 20 Hz or above (see also Fig. 2C, below).

Indeed the incomplete recovery during the 2-s rest interval was expected from a series of earlier studies detailing a RRP recovery time course taking tens of seconds to run to completion when measured with independent techniques, including when using Ca$^{2+}$-independent osmotic shocks instead of electrical stimulation to trigger release (Rosenmund and Stevens 1996; Stevens and Tsujimoto 1995; Stevens and Wesseling 1998; Wesseling and Lo 2002).

Thus for the still depleted synapses to release enough neurotransmitter to generate a fully recovered response to single
pulses, $P_{\text{VES}}$ must have been increased because the probability of release per synapse had completely recovered even though there were fewer readily releasable vesicles (see Fig. 1, A1 and B1). The increase in $P_{\text{VES}}$ would likely pertain to vesicles that had been recruited to the RRP during the previous 2 s because trains of $\geq 60$ pulses (at 20 Hz or faster) are long enough to leave the RRP in a near-empty steady state (Wesseling and Lo 2002).

The increase in $P_{\text{VES}}$ is opposite what would be expected if individual, newly recruited vesicles were primed for release gradually over time intervals lasting $\geq 2$ s. But it is consistent with what would be expected if the residual Ca$^{2+}$ remaining in synaptic terminals after the first train drove an increase in $P_{\text{VES}}$ for the new vesicles that were recruited to the RRP during the interval between trains. Indeed, Ca$^{2+}$ imaging experiments indicated that a small amount of residual Ca$^{2+}$ was still present in axons 2 s after stimulation (Fig. 1C). The increase in $P_{\text{VES}}$ would also explain why response size depressed more quickly during the second train compared to others, documented for a different purpose in Fig. 3 of Wesseling and Lo (2002). The present analysis includes the subset of the previous data that had matching time points, pooled with new data. The subset not included here showed a quantitatively similar effect. (A): fractional recovery vs. length of rest interval for responses to single pulses ($\circ$, as measured as the size of the response to the 1st pulse of the 2nd train divided by the size of the response to the 1st pulse of the 1st train) and the RRP ($\triangle$, measured as described in METHODS). Dashed line, single-exponential function with time constants of 7 s (weighting = 80%) and 30 s (weighting = 20%), chosen to match the decay of residual Ca$^{2+}$ in D. C: frequency jump experiments. After 80 pulses at 20 Hz, stimulation frequency was increased to 40 Hz (gray lines) or maintained at 20 Hz (black lines). Plotted are representative examples of the integral of synaptic responses during 100-ms bins after subtracting responses from identical experiments in the presence of 10 $\mu$M 6,7-dinitroquininaline-2,3-dione (DNQX), scale bar is 25 pC/100 ms bin by 4 s: i: standard conditions: note that no increase in the aggregate response was detected on increasing stimulation frequency; the continuing response after the 60th pulse is proportional to the expected rate of new vesicle recruitment and immediate release (Wesseling and Lo 2002). ii: positive control (0.65 mM Ca$^{2+}$ / 5.0 mM Mg$^{2+}$) where 20-Hz stimulation is no longer sufficient to empty the RRP. Note the robust increase in the response, which indicates an increase in transmitter release. D: clearance of residual Ca$^{2+}$ versus time in Schaffer collaterals after 80 pulses (20 Hz). i: Fluo-4 fluorescence vs. time beginning after 2 s of rest (acquisition at 1 Hz, n = 3 preparations). The white line is a scaled version of the double-exponential function in B. A much larger signal (approximately sixfold, see inset) decayed away much more quickly during the 1st 2 s. Inset: representative example, the gray box indicates analyzed data. The dashed straight line was used as the estimate of signal rundown due to photo-bleaching and was subtracted prior to analysis but was negligible in all cases. Scale is 20% $\Delta F/F$ vs. 20 s. i: single wavelength Furaptra measurements (excitation was $\lambda$ 380 nm); the white line is a scaled and inverted version of same double-exponential function in B.

FIG. 2. $P_{\text{VES}}$ relaxation time course matches Ca$^{2+}$ clearance. A and B: the RRP was emptied with 80 pulses (20 Hz), and subsequent recovery was monitored after an experimentally varied rest interval with an identical train [n = 20 trials from 11 preparations. These experiments had a design that was identical to others, documented for a different purpose in Fig. 3 of Wesseling and Lo (2002). The present analysis includes the subset of the previous data that had matching time points, pooled with new data. The subset not included here showed a quantitatively similar effect.] A: fractional recovery vs. length of rest interval for responses to single pulses ($\circ$, as measured as the size of the response to the 1st pulse of the 2nd train divided by the size of the response to the 1st pulse of the 1st train) and the RRP ($\triangle$, measured as described in METHODS). Dashed line, single-exponential function with time constants of 7 s (weighting = 80%) and 30 s (weighting = 20%), chosen to match the decay of residual Ca$^{2+}$ in D. C: frequency jump experiments. After 80 pulses at 20 Hz, stimulation frequency was increased to 40 Hz (gray lines) or maintained at 20 Hz (black lines). Plotted are representative examples of the integral of synaptic responses during 100-ms bins after subtracting responses from identical experiments in the presence of 10 $\mu$M 6,7-dinitroquininaline-2,3-dione (DNQX), scale bar is 25 pC/100 ms bin by 4 s: i: standard conditions: note that no increase in the aggregate response was detected on increasing stimulation frequency; the continuing response after the 60th pulse is proportional to the expected rate of new vesicle recruitment and immediate release (Wesseling and Lo 2002). ii: positive control (0.65 mM Ca$^{2+}$ / 5.0 mM Mg$^{2+}$) where 20-Hz stimulation is no longer sufficient to empty the RRP. Note the robust increase in the response, which indicates an increase in transmitter release. D: clearance of residual Ca$^{2+}$ versus time in Schaffer collaterals after 80 pulses (20 Hz). i: Fluo-4 fluorescence vs. time beginning after 2 s of rest (acquisition at 1 Hz, n = 3 preparations). The white line is a scaled version of the double-exponential function in B. A much larger signal (approximately sixfold, see inset) decayed away much more quickly during the 1st 2 s. Inset: representative example, the gray box indicates analyzed data. The dashed straight line was used as the estimate of signal rundown due to photo-bleaching and was subtracted prior to analysis but was negligible in all cases. Scale is 20% $\Delta F/F$ vs. 20 s. i: single wavelength Furaptra measurements (excitation was $\lambda$ 380 nm); the white line is a scaled and inverted version of same double-exponential function in B.

The increase in $P_{\text{VES}}$ would explain why response size depressed more quickly during the second train because each pulse would elicit the release of a higher fraction of the available vesicles within the RRP, which would thus be expended sooner.
Relaxation: transient time course for increased $P_{\text{VES}}$

To determine how long $P_{\text{VES}}$ remains increased above baseline during rest, the interval between trains was varied experimentally in a similar series of experiments, as diagrammed at the top of Fig. 2. The relative recovery exhibited by the response to the first pulse of the second train (Fig. 2A, circles) was calculated by dividing the size by the size of the response to the first pulse of the first train, while RRP recovery (Fig. 2A, triangles) was estimated as in Wesseling and Lo (2002); the procedure is also described in METHODS. The increase in $P_{\text{VES}}$ remaining after each rest interval was then quantified by dividing the recovery exhibited by the response to the first pulse of the second train by the RRP recovery (Fig. 2B, note that this calculation yields the relative change in $P_{\text{VES}}$ with respect to baseline, not the absolute value). The analysis showed that the increase in $P_{\text{VES}}$ was transient, returning to baseline with a time course that could be approximated with the same single-exponential function ($\tau = 7$ s) that also characterizes the decay of augmentation as defined previously (Magleby and Zengel 1976a; Stevens and Wesseling 1999a).

Confirmation of RRP exhaustion after 80 pulses

To confirm RRP exhaustion after 80 pulses under our standard conditions, additional experiments were conducted where the stimulation frequency was doubled to 40 Hz after 80 pulses at 20 Hz (Fig. 2C); additionally, identical matched trials were performed in 10 $\mu$M DNQX, which, in combination with the standard NMDA receptor blockers, nearly completely blocks glutamate receptor responses at Schaffer collateral synapses. The complete time-integrated synaptic response was then extracted from these data by binning the entire digitized traces (100-ms bins) after first subtracting the corresponding digitized traces recorded in DNQX. Under standard conditions, no change was detected in the time-integrated synaptic response [Fig. 2Ci], the complete response integral over the first 1 s after doubling the stimulation frequency was 99 $\pm$ 9% ($n = 4$) compared with matched controls where the stimulation frequency was maintained at 20 Hz throughout. In contrast, a robust increase in the time-integrated synaptic response was detected in additional experiments conducted, as a positive control at a highly reduced extracellular Ca$^{2+}$ level where $P_{\text{VES}}$ is lowered to the point where 20-Hz stimulation is no longer sufficient to empty the RRP [Fig. 2Cii, under highly reduced Ca$^{2+}$, low $P_{\text{VES}}$ conditions, doubling the stimulation frequency increased the response integral to 168$\%$ $\pm$ 5% ($n = 3$) of matched controls]. Together these experiments confirm that 80 pulses at 20 Hz leave the RRP in a nearly empty state under standard conditions because otherwise increasing the stimulation frequency would have increased the rate of neurotransmitter release and thus the aggregate postsynaptic response. Thus we reason that the increase in $P_{\text{VES}}$ apparent in Fig. 2, A and B, pertains to vesicles that were recruited to the RRP during the short rest intervals allowed between the paired trains of stimulation.

Residual Ca$^{2+}$ clearance matches relaxation of $P_{\text{VES}}$

As a first test of a working hypothesis that the transient increase in $P_{\text{VES}}$ is caused by the same residual Ca$^{2+}$-dependent mechanism that underlies augmentation induced under other circumstances, fluorescence-imaging experiments were conducted to determine the time course of residual Ca$^{2+}$ clearance from Schaffer collaterals after similar repetitive trains. In separate experiments, time courses were measured with either Fluo-4 or furaptra to control for possible artifacts associated with indicator dye saturation (Sabatini and Regehr 1998) or interference with the natural time course of Ca$^{2+}$ clearance owing to Ca$^{2+}$ buffering by the indicators; these dyes have more than a 100-fold difference in affinity for Ca$^{2+}$ binding (Haugland 1992).

Small, but detectable, amounts of fluorescence remained 2 s after the end of stimulation (16.2 $\pm$ 0.9% of peak for Fluo4, 30.6 $\pm$ 14.7% for furaptra; not significantly different, $n = 3$ for both, see insets of Fig. 2D). Subsequent Ca$^{2+}$ clearance time courses measured with the two dyes were indistinguishable (Fig. 2D) (see Brager et al. 2003 for a quantitatively similar measurement with different techniques) and matched the decay back to baseline in $P_{\text{VES}}$ (Fig. 2E) consistent with the working hypothesis that residual Ca$^{2+}$ increases $P_{\text{VES}}$ of newly recruited vesicles, just as it does for vesicles that have been in the release state for minutes (Stevens and Wesseling 1999a).

No evidence for preferentially faster recruitment of vesicles with high intrinsic $P_{\text{VES}}$

An alternate explanation for the faster recovery of the responses to single pulses would be that after being emptied, the RRP is preferentially restocked first with a few vesicles with intrinsically high $P_{\text{VES}}$, followed later by a larger contingent with a lower $P_{\text{VES}}$. However, previous studies of excitatory hippocampal synapses grown in cell culture showed that RRP recovery after partial depletion of only $\sim$15% proceeded with a time course that was similar to recovery after full RRP depletion, suggesting that the vesicles with the highest $P_{\text{VES}}$ are not restocked more quickly at hippocampal synapses (see Fig. 2 of Stevens and Wesseling 1999a). On the other hand, there is evidence for heterogeneity in the release properties among individual vesicles within the RRP at a large variety of synapse types, including excitatory hippocampal synapses (Hanse and Gustafsson 2001; Millar et al. 2005; Molder and Mennerick 2005; Neher and Zucker 1993). And several reports have suggested recently that the vesicles with a higher $P_{\text{VES}}$ can be restocked at a rate that is different from the average, at least at Calyx of Held synapses, although in those cases it was suggested that the high $P_{\text{VES}}$ vesicles were restocked more slowly than the average (Sakaba and Neher 2001a; Wadel et al. 2007).

It was thus worthwhile to test the synapses used in the present study for possible faster recruitment to the RRP of the vesicles with the highest intrinsic $P_{\text{VES}}$. The experimental design was conceptually similar to the experiments documented in the preceding text, except the first train consisted of only three pulses (Fig. 3A). As in the preceding experiments, the second train consisted of $\geq$60 pulses, which were needed for complete RRP depletion. After the shortest rest intervals, the sum of the responses to the first 60 pulses during the second train was reduced by 18 $\pm$ 0.6% and subsequent recovery of this sum followed a time course that was similar, or slower, than the time course of full RRP recovery (Fig. 3B). As the three pulses in the first train would preferentially expend the vesicles with the highest $P_{\text{VES}}$, these results indicate that high $P_{\text{VES}}$ vesicles are not replaced more quickly than the average.
arguing against the alternate explanation (see DISCUSSION). Further experiments documented below show that synaptic strength transiently overshoots the baseline value under some circumstances (see Fig. 6C), and the overshoot would not be compatible with the alternate explanation either.

**P<sub>VES</sub> relaxation after extensive use continues to match Ca<sup>2+</sup> clearance**

As episodes of extensive use slow the overall rate at which vesicles are recruited to the RRP (Stevens and Wesseling 1999b), an additional set of experiments (diagrammed above Fig. 4) was conducted to determine if this additional component of depression interferes with the increase in P<sub>VES</sub>. No interference was detected after stimulus trains of 600 pulses (Fig. 4A), even though RRP recovery was slower (compare Fig. 4A to Fig. 2A). The time courses of the decay back to baseline of the increase in P<sub>VES</sub> (Fig. 4B) and the clearance of residual Ca<sup>2+</sup> (Fig. 4C) were both lengthened by about twofold but remained matched (Fig. 4D). In addition, three-way comparisons showed no difference in the relationship between intracellular Ca<sup>2+</sup> and P<sub>VES</sub> after 80 and 600 pulses (Fig. 4D). This is a striking result because the electrophysiological subset of the data were extracted from recovery time courses that differed by >10-fold. (The bend in the theoretical curve in Fig. 4D is consistent with partial saturation of the underlying mechanism, see following text).

**Faster relaxation near body temperature matches augmentation**

One characteristic feature of the decay time course of augmentation is its temperature dependence, which was characterized by a Q<sub>10</sub> of 3–4 (Delaney and Tank 1994; Magleby and Zengel 1976a). To determine if the decay time course for P<sub>VES</sub> at recovering synapses is equally sensitive to temperature changes, a series of experiments similar to Fig. 2 was conducted at 33–35°C. The mismatch between the time course of recovery of responses to single pulses and the time course of RRP recovery was even more dramatic at the elevated temperature (Fig. 5A), and P<sub>VES</sub> decayed to baseline more than threefold faster than at room temperature (Fig. 5B, time constant of 2 s; compare with Fig. 2B). The time course of residual Ca<sup>2+</sup> clearance was faster as well (Fig. 5C) and remained matched to the decay of P<sub>VES</sub> (Fig. 5D).

As room temperature was ~10° cooler (23–25°C), these results indicate that the decay time courses for P<sub>VES</sub> and residual Ca<sup>2+</sup> both have Q<sub>10</sub>'s of about 3 at recovering synapses.
apses, matching the temperature sensitivity of augmentation when studied in isolation. More generally, the time course of residual Ca\(^{2+}\) clearance matched the decay of P\(_{\text{VES}}\) to baseline levels even when manipulated experimentally over a ninefold range \(\text{[34°C]}\). After a 2-s rest interval, conditions so that the amount of transmitter release would be first train (3 s) was kept constant for the two experimental series of experiments showed that the 60 pulses that are already driven to a maximal level by the residual Ca\(^{2+}\) accumulates during 60-pulse trains.

**Accretion: saturation of P\(_{\text{VES}}\) increase**

To explore further the relationship between the recovery from depression of responses to single pulses and augmentation, we next examined the dependence on the amount of stimulation. When studied in isolation, augmentation increases steadily with stimulating frequency (Stevens and Wesseling 1999a; Zengel and Magleby 1982), but, in contrast, the next series of experiments showed that the 60 pulses that are required to empty the RRP drive P\(_{\text{VES}}\) to a near-maximal level.

Paired trains were used, similar to the preceding text. For the first set of experiments, the first train of each pair consisted of either 60 pulses at 20 Hz or 120 at 40 Hz; the duration of the first train (3 s) was kept constant for the two experimental conditions so that the amount of transmitter release would be equivalent (Wesseling and Lo 2002). After a 2-s rest interval, the response to the first pulse of the second train was similar for the two conditions (Fig. 6A, actually 12 ± 7.7% smaller after 40-Hz stimulation, but this difference was not statistically significant). Because the RRP recovered slightly less during the 2-s rest interval after 40-Hz stimulation (37 ± 5.2 vs. 43 ± 3.4%, also not significant), the amount of increase in P\(_{\text{VES}}\) was indistinguishable (2.0 ± 0.3-fold vs. 2.0 ± 0.4). Thus although higher frequency stimulation was initially effective at releasing transmitter at a higher rate (Fig. 6A), no additional effect on P\(_{\text{VES}}\) was detected when measured 2 s later.

These data indicate that P\(_{\text{VES}}\) does not always increase linearly with more stimulation, specifically not after 60 pulses with a standard concentration of extracellular Ca\(^{2+}\) (2.6 mM). Although this behavior does not match that of augmentation when studied in isolation from depression, the increase in P\(_{\text{VES}}\) above baseline that is apparent during recovery from depression could still reflect common underlying machinery that was already driven to a maximal level by the residual Ca\(^{2+}\) that accumulates during 60-pulse trains.

The possible presence of a maximal level for P\(_{\text{VES}}\) was tested by varying the Ca\(^{2+}\) and Mg\(^{2+}\) concentrations in the extracellular recording solution. Raising Ca\(^{2+}\), or lowering Mg\(^{2+}\), increases the baseline synaptic response because more Ca\(^{2+}\) flows into the terminals with each action potential, thus increasing the baseline value of P\(_{\text{VES}}\) (Augustine and Charlton 1986; Dodge and Rahamimoff 1967; Fernandez-Chacon et al.

**FIG. 5.** P\(_{\text{VES}}\) relaxation and Ca\(^{2+}\) clearance remain matched near body temperature. A and B: the RRP was emptied with 80 \((n = 6\) trials from 3 preparations) or 120 pulses \((n = 4\) trials from 3 preparations), and subsequent recovery was monitored after an experimentally varied rest interval with 80 pulses (all stimulation at 40 Hz). No differences were detected in recovery values between the 2 types of experiments, and so the data were pooled. A: fractional recovery vs. rest interval for both the response to single pulses (circles) and the entire RRP (diamonds). The dashed line is Eqn 2 from Wesseling and Lo (2002) where \(\alpha(t)\) starts off at 0.4 /s and decays exponentially 3.7-fold with a 2-s time constant. Note the nonmonotonic recovery of responses to single pulses. The gray boxes indicate measurements after the same rest interval, which are compared in B. B: decay vs. time (i.e., rest interval) of P\(_{\text{VES}}\) calculated by dividing single pulse response recovery values by RRP recovery values (both displayed in A). The dashed line is an exponential function with a time constant of 2 s. C: clearance vs. time of residual Ca\(^{2+}\) after 60 pulses (40-Hz stimulation, 33–35°C, Fluo-4, detection at 5 Hz, \(n = 3\) preparations). The white line is a scaled version of the same exponential function as in B. Data points are normalized by the response size after the 1st 1 s of rest. Inset: example, scale is 20% ΔF/IP vs. 5 s. D: comparison between the Fluo-4 signal and changes in P\(_{\text{VES}}\).
2001). But, if \( P_{\text{VES}} \) already approached a maximal level during the 60-pulse train under standard conditions, raising the \( \text{Ca}^{2+}/\text{Mg}^{2+} \) ratio would no longer be expected to have as much impact.

Raising the \( \text{Ca}^{2+}/\text{Mg}^{2+} \) ratio did indeed have a reduced impact on synaptic responses at times when \( P_{\text{VES}} \) had already been increased (Fig. 6B). The response to the first pulse of the first train was \( 1.77 \pm 0.094 \)-fold larger at the higher \( \text{Ca}^{2+} \) concentration (4.5 mM, Fig. 6Bii). But the response to the first pulse of the second train was significantly less affected \( (P < 0.02) \); i.e., only \( 1.20 \pm 0.17 \)-fold larger in high \( \text{Ca}^{2+} \) (Fig. 6Biii). Because the RRP recovered to a similar extent during the first train was 1.77 \( \pm 0.094 \)-fold larger (Fig. 6Bii).

Nevertheless, the theoretical curve in Fig. 4D, and the trend for the response to the first pulse of the second train to be somewhat larger at 4.5 mM \( \text{Ca}^{2+} \) versus at 2.5 mM \( \text{Ca}^{2+} \) (Fig. 6Bii) suggested that any underlying saturation of the release trigger may not have been complete after 60 pulses under the standard conditions used in the preceding text. Analysis of additional, similar experiments making up a larger data set confirmed this, having the resolution to show that the first response of the second train was \(-25\%\) higher at the higher \( \text{Ca}^{2+} \) level, and that the induction of depression was correspondingly faster (Fig. 6Biii).

**FIG. 6.** Activity drives \( P_{\text{VES}} \) to a near-maximal level. A: number of pulses: the RRP at Schaffer collateral terminals was emptied twice in succession with pairs of repetitive trains as diagrammed at top \( (n = 9 \) paired trials from 3 preparations). The 1st train consisted of either 60 (20 Hz) or 120 (40 Hz) pulses over 3 s. After a 2-s rest interval, the 2nd train consisted of 80 pulses (20 Hz). i: overlaid averaged traces of responses during the 1st pulse of 1st train. Gray traces are 40-Hz stimulation, black are 20 Hz. Note that 40-Hz stimulation was initially effective at releasing transmitter at a higher rate. ii: overlaid averaged traces of responses to the 1st pulse of the 2nd train. Note that these responses were similar in size indicating that 40-Hz stimulation did not drive \( P_{\text{VES}} \) to a substantially higher level. Scale is 200 pA vs. 40 ms. iii: plot of response sizes during the 2nd train for both conditions (normalized by the response size after the 1st pulse of the 1st train). Note that no difference was detected in the decay. B: \( \text{Ca}^{2+} \) concentration: i and ii. The RRP was emptied with 80 pulses (20 Hz) and recovery was monitored after a 2-s rest interval with a similar train as diagrammed at top with either a normal (2.5 mM/2.5 mM) or high (4.5 mM/0.5 mM) ratio of extracellular \( \text{Ca}^{2+}/\text{Mg}^{2+} \) \( (n = 7 \) paired trials from 3 preparations); the total divalent ion concentration was held constant to avoid large changes in axonal excitability that might affect the number of different synapses activated with each pulse (Frakenhaeuser and Hodgkin 1957). i: averaged traces of responses to the 1st pulse of the 1st train for the 2 conditions; the stimulation artifact is blanked. Note that the response was substantially larger at the higher \( \text{Ca}^{2+} \) concentration. ii: averaged responses to the 1st pulse of 2nd train. Note that increasing \( \text{Ca}^{2+} \) had a reduced impact on these responses. Scale is 100 pA vs. 20 ms. Changes in response size during the 2nd train for a larger data set from similar experiments; the high \( \text{Ca}^{2+}/\text{Mg}^{2+} \) ratio \( (n = 41 \) trials, 9 preparations) was either 4.5 mM/0.5 mM, or 5.0 mM/0.5 mM, and standard was 2.6 mM/1.3 mM \( (n = 31 \) trials, 20 preparations). For these plots, response sizes were normalized by the mean size of responses to pulses 61–80 of the 1st train because this measure is not affected by changing \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \) levels (Wesseling and Lo 2002). Although error bars were omitted for clarity, the response to the 1st pulse of the 2nd train was significantly larger in high \( \text{Ca}^{2+} \) \( (P < 0.05) \), and responses to pulses 15–45 were significantly smaller, indicating that depression was induced faster and that \( P_{\text{VES}} \) was not completely maximal 2 s after 80 pulses under standard conditions. C: synaptic strength overshoots baseline at reduced extracellular ratio of \( \text{Ca}^{2+}/\text{Mg}^{2+} \) \( (1.3 \text{ mM}/2.6 \text{ mM}) \) during RRP recovery. Responses were evoked with pairs of 20-Hz trains separated by a 5-s rest interval \( (n = 18 \) trials from 3 cells). i: averaged traces of responses to the 1st pulse of each train as indicated (stimulus artifacts are blanked). Note that the response to the 1st pulse of the 2nd train was substantially larger than the response to the 1st pulse of the 1st train. Scale is 50 pA by 25 ms. ii: response sizes vs. time for the entire experiment (normalized by the response size after the 1st pulse of the 1st train). The dashed lines represent the fraction of each response that is due to the release of vesicles that became available for release during each stimulus train according to a working model of RRP recovery described in the APPENDIX of (Wesseling and Lo 2002). Initial \( P_{\text{VES}} \) calculated from these data, with the model was 1.8\% (compare with 4.4\% under standard conditions) and the rate of new vesicle recruitment to the RRP was 0.26/s (matching the value under standard conditions).
Synaptic strength overshoots in lower Ca\(^{2+}\)

At the standard Ca\(^{2+}\) and Mg\(^{2+}\) concentrations used in the preceding text, responses to single pulses recovered to the baseline value more quickly than did the entire RRP but never surpassed the baseline by a substantial amount. If this is because of a limit on the maximum value of P\(_{\text{VES}}\), it should be possible to design an experiment where the responses to single pulses do overshoot baseline after the induction of depression by, for example, lowering the baseline value of P\(_{\text{VES}}\) and thus increasing the available dynamic range.

A large amount of overshoot in the response to single pulses could indeed be achieved by lowering the Ca\(^{2+}/\)Mg\(^{2+}\) ratio. After a 5-s rest interval in 1.3 mM Ca\(^{2+}/2.6\) mM Mg\(^{2+}\), the response to the first pulse of the second train was 2.13 ± 0.36-fold larger than the response to the first pulse of the first train (Fig. 6C, i and ii). Additional control experiments indicated that the first train was still sufficient to empty the RRP at the lower Ca\(^{2+}\) level (Supplementary Fig S1).

As noted earlier, the overshoot is predicted by the working model, but is incompatible with alternatives that would suppose that the mismatch between the time course of recovery of responses to individual pulses and the time course of RRP recovery arises instead from different replenishment rates of independent subdivisions of the RRP.

Cell culture measurements in the presaturating range

It was not feasible to measure the effect of fewer than 60 pulses on P\(_{\text{VES}}\) for newly recruited vesicles in the slice preparation because the basic experimental design requires that the first train empty the RRP. To link the fast recovery of single pulse responses to previously defined augmentation, it was nevertheless important to determine if the increase in P\(_{\text{VES}}\) requires action potentials and residual Ca\(^{2+}\) or if it is instead some intrinsic feature of newly recruited vesicles.

To circumvent the technical limitation, the RRP can be emptied in a Ca\(^{2+}\)-independent fashion by using osmotic shocks instead of action potentials (Molder and Mennerick 2005; Rosenmund and Stevens 1996). Osmotic shocks are typically induced by flowing hypertonic solutions over synapses, and it was not feasible to temporally and spatially restrict solution exchanges to electrically activated synapses in the slice preparation as would be required. However, it is possible to grow isolated hippocampal neurons in primary culture where solution exchanges can be performed quickly and with more spatial precision (Bekkers and Stevens 1991; Rosenmund and Stevens 1996). Isolated neurons have the additional advantage that they form recurrent synaptic connections (autapses), making it uniquely possible to simultaneously elicit exocytosis with osmotic shocks and action potentials at the same afferent synapses (Rosenmund and Stevens 1996; Stevens and Wesseling 1998).

Pilot experiments showed that autapses retain the mismatch between the fast recovery of the responses to single pulses, and the slower RRP recovery time course (data not shown), permitting us to combine osmotic shocks with electrical stimulation to determine the effects of small numbers of presynaptic action potentials on P\(_{\text{VES}}\) during RRP recovery. The experimental design is diagrammed at the top of Fig. 7. For each experiment, the RRPs were emptied twice in succession with pairs of osmotic shocks separated by 5-s rest intervals, and single-pulse responsiveness was monitored with “test” action potentials elicited 100 ms before each osmotic shock. Activity dependent enhancement of the single pulse response preceding the second osmotic shock (i.e., “test AP 2” in the diagram above Fig. 7) was achieved by evoking between 0 and 40 “activating” action potentials during the last 1 s of the first osmotic shock (Fig. 7, A and B). As some of the increase in the single pulse responses could have been due to an increase in the number of vesicles recruited to the RRP (Stevens and Wesseling 1998), the increase in RRP recovery was also estimated by comparing the response to the second osmotic shock to the response to the first osmotic shock but was substantially less (Fig. 7B). Increases in P\(_{\text{VES}}\) (relative to baseline) were then extracted from these measurements by dividing the amount of increase in the response to test AP 2 by the amount of increase in RRP recovery (Fig. 7B). The analysis showed that P\(_{\text{VES}}\) increased monotonically with the first 20 activating action potentials with a characteristic accretion that could be approximated by a single exponential with an exponential parameter of nine action potentials (Fig. 7B, see legend for equation).

Ca\(^{2+}\) dependence

The experiments summarized in Fig. 7, A and B, were conducted at a low Ca\(^{2+}/\)Mg\(^{2+}\) ratio (1 mM/3 mM) to set the baseline value of P\(_{\text{VES}}\) far from the maximum level, but the effect was also present after 2-s rest intervals with the standard concentrations of Ca\(^{2+}\) and Mg\(^{2+}\) used in the slice preparation (2.6 mM/1.3 mM). In these additional experiments, 40 activating action potentials enhanced the synaptic response to the second test action potential 4.1-fold, while increasing the amount of RRP recovery by only 1.5-fold, for an extrapolated 2.7-fold increase in P\(_{\text{VES}}\).

The techniques available in cell culture allowed a more direct test of the residual Ca\(^{2+}\) dependence of the transient increase above baseline in P\(_{\text{VES}}\) at recovering synapses by using exogenous Ca\(^{2+}\) buffers. The increase that was evident after 2-s rest intervals under standard conditions was nearly completely abolished after 5 min exposure to 100 μM EGTA-AM (Fig. 7C, same neurons, P < 0.05), providing compelling evidence that residual Ca\(^{2+}\) drives the increase in P\(_{\text{VES}}\) for newly recruited vesicles, just as it does to enhance synaptic strength at synapses where the RRP is full, and the release ready vesicles are known to be in the fully primed state.

Potentiated propensity for fusion of newly recruited vesicles

Even when applied rapidly, it takes several hundred milliseconds before osmotic shocks begin to elicit transmitter release, and the length of this delay is thought to reflect the propensity with which readily releasable vesicles can be triggered for exocytosis (Basu et al. 2007; Stevens and Wesseling 1999a). As a final check of the working hypothesis, it was important to determine if this delay is decreased by residual Ca\(^{2+}\) at times when the RRP consists only of newly recruited vesicles because residual Ca\(^{2+}\) is already known to drive a decrease in the delay when the RRP is full of vesicles that have been in the releasable state for minutes. Although designed and
originally analyzed to answer a different question (Stevens and Wesseling 1998), experiments that test this have already been conducted in cell culture (Fig. 8A). A more extensive analysis of the previous data set showed that, indeed, the residual Ca\(^{2+}\)-dependent increase in \(P_{\text{VES}}\) at recovering synapses is associated with a decrease in the delay before onset of the synaptic response to osmotic shocks (Fig. 8, B and C, left). The decrease was similar and has a similar time course of decay (Fig. 8D), to the decrease at synapses where the readily releasable vesicles had been recruited \(\geq 1\) min earlier (Stevens and Wesseling 1999a). Further, EGTA blocked the decrease in the delay indicating that the decrease depended on residual Ca\(^{2+}\) (Fig. 8, B and C, right).

In contrast, in the absence of action potentials, no countervailing increase in the delay was detected, even in the presence of EGTA (Fig. 8C, right). The lack of an increase is relevant because one might have been expected if individual vesicles were primed for release in a graded fashion over a time course of seconds or minutes.

**Occlusion of paired-pulse facilitation**

Under the low \(P_{\text{VES}}\) conditions of previous experiments, augmentation has been shown to operate independently of other elements of enhancement such as paired-pulse facilitation (PPF) (Magleby and Zengel 1976a; Zengel and Magleby 1982). Despite the mechanistic independence, however, our results predict that PPF should be occluded by augmentation at times when \(P_{\text{VES}}\) is already nearly maximal because PPF is also expressed as an increase in \(P_{\text{VES}}\). As occlusion could have important physiological implications, the electrophysiological data documented in Figs. 2 and 4 were reanalyzed to determine the extent of PPF during recovery intervals. The expression of PPF was indeed abolished for the first several seconds after stimulation, and this interference dissipated with a time course that was similar to the decay of \(P_{\text{VES}}\) (Fig. 9, A and B). PPF was also transiently abolished in the near-body temperature experiments, but the baseline amount was too small (\(~15\%) to extract a clear recovery time course from the data set documented in Fig. 5. Additional experiments were thus conducted.

FIG. 7. Residual Ca\(^{2+}\)-dependent increase in \(P_{\text{VES}}\) in hippocampal cell culture autapses after emptying the RRP with osmotic shocks. A and B: recovery of single pulse responsiveness was measured after emptying the RRP with osmotic shocks, as diagrammed at top. A variable number of presynaptic “activating” action potentials (APs) was evoked during the last 1 s of the 1st osmotic shock, and the impact on responses to single pulses (test AP 2) and PPF recovery (2nd osmotic shock) was monitored 5 s later (extracellular Ca\(^{2+}\)/Mg\(^{2+}\) was 1 mM/3 mM). A: example of the impact of activating APs on single pulse responses [i.e., responses to test AP 2; same neuron, change in baseline size (2nd osmotic shock)] by the relative increase in \(P_{\text{VES}}\) (Fig. 9, A and B). B: PPF was indeed abolished for the first several seconds after stimulation, and this interference dissipated with a time course that was similar to the decay of \(P_{\text{VES}}\) (Fig. 9, A and B). PPF was also transiently abolished in the near-body temperature experiments, but the baseline amount was too small (\(~15\%\) to extract a clear recovery time course from the data set documented in Fig. 5. Additional experiments were thus conducted.

\[y = (1 - e^{-0.5 \times 30}) + 1.5 + 0.5\]

C: enhancement of \(P_{\text{VES}}\) is blocked by EGTA. These experiments were similar to the ones documented in A and B except extracellular Ca\(^{2+}\)/Mg\(^{2+}\) was 2.6 mM/1.3 mM, the interval between osmotic shocks was 3 s, and only trials with 40 and 0 activating APs were conducted (paired experiments on 4 neurons, *\( = P < 0.05\)). Fractional recovery was calculated as the size of the response to the test AP 2 divided by the size of the response to test AP 1.
at a reduced extracellular Ca\(^{2+}\) level (1.6 mM instead of 2.6 mM), and the resulting recovery time course (Fig. 9C) matched the decay in \(P_{\text{VES}}\) at the elevated temperature as well (compare with Fig. 5B). In this context, it is notable that robust PPF was present 2 s after the three-pulse trains used to partially deplete the RRP (see Fig. 3) in agreement with Fig. 7B, which indicates that three action potentials are not enough to drive \(P_{\text{VES}}\) to the maximum level.

**FIG. 8.** Activity drives a decrease in the delay before response onset during osmotic shocks for newly recruited release ready vesicles. Reanalysis of data from Stevens and Wesseling (1998). A–C: the RRP was emptied with pairs of osmotic shocks with 2-s rest intervals between shocks. Thirty activating APs were included at the end of the 1st osmotic shock for experimental conditions, and omitted in controls. A: averaged raw data traces from the experiments where data were also available after treatment with EGTA (3 autapses). Left: traces are prior to treatment with EGTA; right, traces are afterward. Traces from experimental (with APs) and control (without APs) conditions are overlaid in each case. Note that the most striking effects of APs in these traces are the increased steady state release rate during the 1st osmotic shock, and the larger response during the 2nd; both of these effects are thought to reflect residual Ca\(^{2+}\)-dependent acceleration of new vesicle recruitment to the RRP, which has been reported previously (Stevens and Wesseling 1998). Inset: scaled traces of the rising phase of the response to the 2nd osmotic shocks to show that the timing of release is also affected. B: binned responses (50 ms/bin) of data from A showing the delay and rising phase of the response during the 2nd osmotic shock. For comparison, responses are normalized by the peak bin size of each response. Note that APs decreased the delay before the onset of the response and that EGTA blocked this effect. C: binned responses from A comparing rising phases of the responses during the 1st and 2nd osmotic shocks when APs were included at the end of the 1st osmotic shock. D: decrease in delay before response onset vs. duration of rest interval. The RRP was dumped with osmotic shocks twice in succession as above, except only 20 APs were included at the end of the 1st osmotic shock for all trials, and the rest interval was varied experimentally. Decreases in the delay before response onset were quantified by subtracting the length of the delay during the 2nd osmotic shock from the length of the delay during the 1st (\(n = 5\) neurons, \(\approx 1\) trial for each interval per cell). - - - : exponential function with a time constant of 7 s, which matches the decay of augmentation (Stevens and Wesseling 1999a).

**DISCUSSION**

Short-term synaptic plasticity includes both positive and negative components, but the kinetic rules by which the components interact have been largely unexplored (Kalkstein and Magleby 2004; Korn and Faber 1987; Zucker 1973). The positive components are known collectively as short-term enhancement. They are mostly driven by residual Ca\(^{2+}\) and include several kinetically distinct elements (Fisher et al. 1997). One element, previously termed *augmentation*, persists for several seconds owing to slow residual Ca\(^{2+}\) clearance from presynaptic terminals and is expressed as an increase in the fraction of the available vesicles within the RRP that is released following single action potentials; i.e., as an increase in \(P_{\text{VES}}\) (Stevens and Wesseling 1999a).

We show here that residual Ca\(^{2+}\) continues to increase \(P_{\text{VES}}\) via the same or similar mechanism at excitatory hippocampal synapses at times when RRP replenishment is ongoing and the overall probability of release is depressed due to RRP depletion. 1) Single action potentials trigger the release of a larger fraction of the newly recruited readily releasable vesicles during recovery from depression than they do under baseline conditions (i.e., \(P_{\text{VES}}\) is increased, Figs. 1 and 7B). 2) The increase in \(P_{\text{VES}}\) requires residual Ca\(^{2+}\) (Fig. 7C). 3) The increase in \(P_{\text{VES}}\) is transient, decaying away with a time course that matches the clearance of residual Ca\(^{2+}\) even when the time course is manipulated experimentally over a ninefold range (Figs. 2, 4, and 5). And 4), the increase in \(P_{\text{VES}}\) occurs in concert with a decrease in the delay before onset of neurotransmitter release when the release is triggered with osmotic shocks instead of action potentials, suggesting that the energy barrier that normally prevents fusion is lowered (Fig. 8).

Alternative models that ascribe the increase in \(P_{\text{VES}}\) during recovery from depression to a different type of mechanism than the one underlying augmentation could account for some of our
observations, but would need to be complicated to account for all of them. For example, the nonmonotonic recovery of single pulse responses in Fig. 5A, the overshoot above baseline in single pulse responses in Fig. 6C, and the absence of a faster RRP recovery time course after partial depletion (see Fig. 3 of the present manuscript, and Fig. 2 of Stevens and Wesseling 1999a) would rule out alternatives whereby a highly releasable subset of the RRP is replenished first, followed later by reluctantly releasable vesicles.

The presence of residual Ca\(^{2+}\) does not necessarily cause synaptic strength to enhance above baseline, however, because residual Ca\(^{2+}\) levels are typically highest immediately after active episodes when the RRP tends to be most depleted, and by the time the RRP has fully recovered, residual Ca\(^{2+}\) clearance is complete. Residual Ca\(^{2+}\), instead, accelerates recovery from depression in the single pulse release probability so that it considerably outpaces RRP recovery (Fig. 10). Thus a fast rebound from depression often does not indicate that synapses are quickly restored to their initial functional state because the probability of release depresses more quickly during subsequent bursts of activity.

Alternate definitions for the RRP

A slower decay for enhancement (Klyachko and Stevens 2006) and a delay between the clearance of residual Ca\(^{2+}\) and the decay of enhancement (Brager et al. 2003) have been reported previously for excitatory hippocampal synapses, but no such delay was apparent in our experiments. The discrepancies could be due to differences in the interpretation of data.

One key difference is that Brager et al. and Klyachko and Stevens both judged that RRP replenishment was complete at times when depression was no longer evident in the responses to single pulses. However, a re-analysis of their data that takes account of the slower RRP replenishment time courses reported here and elsewhere (Rosenmund and Stevens 1996; Stevens and Tsujimoto 1995; Wesseling and Lo 2002) yield a faster decay for enhancement, which then does match the recovery of single pulse release probability (Fig. 10). Model: The dotted line represents the decay of underlying augmentation (residual Ca\(^{2+}\) driven increases in \(P_{\text{ves}}\), and the dashed line is the RRP recovery time course. The solid gray line is the product of these underlying elements and represents the recovery of single pulse release probability. The theoretical curves representing component elements were chosen to match parameters measured near body temperature (see Fig. 5, A and B). The multi-tonic recovery time course of the single pulse release probability is reminiscent of early reports of a similar phenomenon at neuromuscular junctions (Liley and North 1953).
clearance of residual Ca\(^{2+}\), at least in the case of Brager et al., who also measured the residual Ca\(^{2+}\) clearance time course.

Thus some of our quantitative conclusions depend on our observation that the RRP takes longer to replenish than has been reported elsewhere. Our comparatively slow estimates of RRP replenishment rates are based on a variety of independent techniques, including pairs of osmotic shocks, which elicit transmitter release downstream of action potentials and Ca\(^{2+}\) influx (Rosenmund and Stevens 1996; Stevens and Tsujimoto 1995), pairs of 60-pulse trains of electrical stimulation (Wesseling and Lo 2002), and combinations of electrical stimulation and osmotic shocks (Figs. 7 and 8) (see also Stevens and Wesseling 1998, 1999b). All of these types of measurements yield a RRP size and recovery time course that 1) are consistent across experimental conditions, 2) do not change when the extracellular Ca\(^{2+}\) level is changed, even while synaptic responses to single pulses change dramatically, and 3) are similar or identical in cell culture and hippocampal slices (Rosenmund and Stevens 1996; Stevens and Williams 2007; Wesseling and Lo 2002).

On the other hand, faster estimates of RRP replenishment at comparable synapses have typically been derived from measurements of recovery from depression of the responses to single pulses or short trains of stimulation, and this measurement strategy can be compromised by residual Ca\(^{2+}\) dynamics. That is, a key prediction of the present study is that recovery time courses measured with short-train stimulation protocols (or single pulses) would be faster than the underlying recruitment of new vesicles if the short trains were not sufficient to empty the RRP because a larger fraction of the readily releasable vesicles would be released after brief rest intervals, before clearance of the residual Ca\(^{2+}\), than after longer rest intervals. Indeed we can confirm from our own data that recovery time courses measured with short trains are faster than time courses measured with our standard 60-pulse stimulation protocol, falling somewhere between the pairs of recovery time courses plotted in Figs. 2A, 4A, and 5A (not shown).

In this context, we make the perhaps counterintuitive prediction that estimates of the timing of RRP recovery that are based on short-train protocols would appear to be substantially slower at high extracellular Ca\(^{2+}\) levels, or under other conditions where baseline P\(_{\text{VES}}\) is higher, because under high baseline P\(_{\text{VES}}\) conditions, short trains would be more effective at emptying the RRP, which would avoid the confounding effect of residual Ca\(^{2+}\). And, indeed, this prediction seems to hold in several recent studies (Molder and Mennerick 2005; Schluter et al. 2006; Toonen et al. 2006).

Relation to a “preprimed pool”

Our study does not rule out the presence of an immediately releasable pool (IRP), or preprimed pool, that would be a subset of the RRP by our definition (Hanse and Gustafsson 2001; Sakaba and Neher 2001b). If present, such a pool would be replenished with vesicles either 1) from the greater RRP, i.e., the RRP by the present definition, or 2) from some other source. In either case, our main conclusions would remain valid for the following reasons. 1) If replenishment were to come from within the greater RRP, then our results could be reinterpreted as reflecting Ca\(^{2+}\) driven dynamic modulation of the size of the hypothetical IRP, but alternate mechanisms such as these would account equally well for synaptic enhancement defined under low probability of release conditions. 2) If the hypothetical IRP were replenished from some other source, the results in Fig. 3 indicate that recovery would proceed with a time course that is similar to that of the greater RRP. Thus, the calculations of increases in P\(_{\text{VES}}\) described above would be similar, and the conclusions would be the same.

In either case, the observation that responses depress to a steady-state level more quickly during a second train started after a 2-s rest interval than during the preceding first train (Fig. 1A) suggest that residual Ca\(^{2+}\) enhances the release efficiency of all readily releasable vesicles. Indeed data from cell culture suggest that reluctantly releasable vesicles may exhibit even more enhancement than the highly releasable ones because during osmotic shocks, residual Ca\(^{2+}\) decreases the delay before release of the last to be released vesicles by more than the delay before release of the first ones (see Fig. 4 of Stevens and Wesseling 1999a).

**Gradual versus stepwise vesicle priming: comparison to the Calyx of Held**

Although it often takes tens of seconds or longer for spent vesicles to be re stocked within the RRP, the present findings imply that individual vesicles make the final transition from not-at-all available to fully primed more quickly, at least at excitatory hippocampal synapses; i.e., in <1 s, but possibly faster. A study of Calyx of Held synapses has suggested that the underlying vesicle priming reaction is gradual because individual, newly recruited vesicles may be only reluctantly releasable, each taking several tens of seconds to achieve a fully primed state (Wu and Borst 1999; but see Wadel et al. 2007). Gradual priming does not appear to be due to a general molecular limitation of vesicle trafficking, however, as we find that readily releasable vesicles are more, rather than less, easily triggered to undergo exocytosis within 1 s after being recruited to the RRP at hippocampal synapses. While our data indicate that the heightened propensity for release of newly recruited vesicles is dependent on the residual Ca\(^{2+}\) that accumulates during the stimulation used to empty the RRP, the osmotic shock experiments summarized in Fig. 8 suggest that even in the absence of residual Ca\(^{2+}\), individual vesicles make a quick transition from completely unavailable to fully releasable. Definitively ruling out a small contribution of gradual vesicle priming was not feasible with the present techniques, however, because in addition to eliciting exocytosis, osmotic shocks also transiently reduce P\(_{\text{VES}}\) (Supplementary Fig S2), possibly by inhibiting Ca\(^{2+}\) channels (Rosenmund and Stevens 1996).

The experimental protocols typically used to exhaust the RRP at the Calyx of Held elicit release much more rapidly than has been possible in our experiments at hippocampal synapses, and it is possible that it is this experimental difference that affects whether or not newly recruited vesicles prime for release gradually and over what time course, but, on the other hand, the physiological requirements for synaptic transmission at the Calyx of Held are exceptionally different from requirements at Schaffer collaterals (Hermann et al. 2007; Ranck 1973), and so the presence of basic differences in the mechanisms underlying short-term plasticity are not necessarily surprising.
In addition, another series of studies of the Calyx of Held has suggested that $P_{\text{VES}}$ may be partly determined by recruitment of Ca$^{2+}$ channels to release sites, which is not complete until around 1 s after priming (Wadel et al. 2007). A similar mechanism would have gone undetected in the present study because rest intervals of <1 s were not tested. In any case, it would be technically difficult to detect the presence of such an event at hippocampal synapses with presently available techniques because the minimum amount of electrical stimulation required to empty the RRP already results in enough residual Ca$^{2+}$ to drive $P_{\text{VES}}$ to a maximum value.

**Mechanism**

The molecular mechanism underlying augmentation remains to be elucidated. Facilitation, which is defined as a much more transient component of short-term enhancement, is thought to depend indirectly on residual Ca$^{2+}$ due to partial saturation of endogenous Ca$^{2+}$ buffers rather than directly via activation of a bona fide residual Ca$^{2+}$ sensor (Felmy et al. 2003), at least in some cases (Zucker 2003). However, an analogous explanation for augmentation would not account for the decrease in the delay before onset of synaptic responses to osmotic shocks (e.g., Fig. 8) because osmotic shocks trigger neurotransmitter release by a mechanism that does not depend on the Ca$^{2+}$ buffering capacity within the synaptic terminals (Rosenmund and Stevens 1996). Also, the Ca$^{2+}$-dependent mechanism underlying augmentation would likely be distinct from the one that triggers exocytosis directly as the sensor is thought to bind Ca$^{2+}$ with higher affinity and a slower dissociation rate (Kamiya and Zucker 1994), and it seems to function as a linear Ca$^{2+}$ detector at the lowest Ca$^{2+}$ concentrations, while the exocytic trigger likely requires the cooperative binding of multiple Ca$^{2+}$ ions (Zucker and Regehr 2002). An intriguing role in controlling $P_{\text{VES}}$ at a variety of synapse types has recently been proposed for diacylglycerol-dependent activation of PKC and/or Munc 13–1 that might be related (Basu et al. 2001; Stevens and Wesseling 2000; Markram and Tsodyks 1996); the terminology used was redistribution of synaptic efficacy. This report shows how these two parameters can also be controlled dynamically and differentially over the several seconds it takes for synapses to recover during rest intervals that routinely separate bursts of action potentials in the Schaffer collaterals of awake and behaving animals (O’Keefe and Dostrovsky 1971; Ranck 1973). Residual Ca$^{2+}$ causes the initial probability of release to recover from depression considerably more quickly than the RRP, but the same synapses then depress more rapidly during subsequent bursts that are initiated after short rest intervals. In this context, classically defined augmentation could be considered to function as a medium-term modulator of the short-term synaptic plasticity exhibited during bursts—i.e., meta short-term plasticity—and would be the second type of residual Ca$^{2+}$-dependent mechanism known to operate at the synaptic level to counter short-term depression, contrasting importantly with mechanisms that accelerate vesicle recruitment and thus hasten the recovery of all parameters to full functionality (Dittman and Regehr 1998; Sakaba and Neher 2001a; Stevens and Wesseling 1998; Wang and Kaczmarek 1998).

**Saturating increases in $P_{\text{VES}}$ cause augmentation to occlude PPF**

There is one notable difference between the generalized residual Ca$^{2+}$ driven enhancement of the release machinery characterized here and augmentation by its original definition. Under standard conditions, it seems that $P_{\text{VES}}$ can be driven to a maximum level at excitatory hippocampal synapses, whereas under the low-Ca$^{2+}$ conditions of the original experiments, no limit has been reported. The presence of a maximum level could have important implications for understanding the roles of other elements of short-term enhancement as well. For example, our data reveal that the expression of PPF is occluded for several seconds after moderate amounts of activity (Fig. 9) (see also Brager et al. 2003; McNaughton 1982). This occlusion is particularly striking, given that it tends to occur at synapses when they are depressed because of the contrast with the current concept that the magnitude of PPF tends to be inversely proportional to release probabilities at fully rested synapses (Dobrunz and Stevens 1997; Hanse and Gustafsson 2002; Manabe et al. 1993).

The cause for the maximal value for $P_{\text{VES}}$ is presently unclear but might be directly due to a limit in the modulatory range of some component of the release machinery or to a secondary effect of partial RRP depletion, perhaps because of depletion of a highly releasable subset of the RRP (i.e., an IRP, see preceding text). On one hand, a twofold increase under standard conditions seems to be near-maximal, which might suggest that the maximum value of $P_{\text{VES}}$ is only ~10–15% because the starting value is only ~5% (Wesseling and Lo 2002). On the other hand, it is likely that some of the individual vesicles within the RRP have a higher intrinsic $P_{\text{VES}}$ than others (Hanse and Gustafsson 2001; Molder and Mennerick 2005). If baseline $P_{\text{VES}}$ of these vesicles were much higher than the average, a twofold potentiation at the start of a second train could cause immediate depletion of this highly releasable subdivision of the RRP, which could explain the apparent maximum value for $P_{\text{VES}}$ of the population average seen here.

**Meta short-term plasticity**

Current theories emphasize the importance of both the initial probability of release and the rate of depression for filtering and transforming information encoded within bursts of action potentials (Chance et al. 1998; Fuhrmann et al. 2002; Natschlager et al. 2001; Tsodyks and Markram 1997). In fact, long-term plasticity of these parameters has been described (Abbott and Nelson 2000; Markram and Tsodyks 1996); the terminology used was redistribution of synaptic efficacy. This report shows how these two parameters can also be controlled dynamically and differentially over the several seconds it takes for synapses to recover during rest intervals that routinely separate bursts of action potentials in the Schaffer collaterals of awake and behaving animals (O’Keefe and Dostrovsky 1971; Ranck 1973). Residual Ca$^{2+}$ causes the initial probability of release to recover from depression considerably more quickly than the RRP, but the same synapses then depress more rapidly during subsequent bursts that are initiated after short rest intervals. In this context, classically defined augmentation could be considered to function as a medium-term modulator of the short-term synaptic plasticity exhibited during bursts—i.e., meta short-term plasticity—and would be the second type of residual Ca$^{2+}$-dependent mechanism known to operate at the synaptic level to counter short-term depression, contrasting importantly with mechanisms that accelerate vesicle recruitment and thus hasten the recovery of all parameters to full functionality (Dittman and Regehr 1998; Sakaba and Neher 2001a; Stevens and Wesseling 1998; Wang and Kaczmarek 1998).


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