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Effects of increasing Ca^{2+} channel-vesicle separation on facilitation at the crayfish inhibitory neuromuscular junction

Tariq N. Allana and Jen-Wei Lin

Department of Biology, Boston University, Boston, MA 02215

Abstract

We investigated the mechanism of facilitation at the crayfish inhibitory neuromuscular junction before and after blocking P-type Ca^{2+} channels. P-type channels have been shown to be closer to releasable synaptic vesicles than non-P-type channels at this synapse. Prior to the block of P-type channels, facilitation evoked by a train of 10 action potentials at 100 Hz was increased by application of 40 mM $[\text{Mg}^{2+}]_o$, but decreased by pressure injected EGTA. Blocking P-type channels with 5 nM ω -Aga IVA, which reduced total Ca^{2+} influx and release to levels comparable to those recorded in 40 mM $[\text{Mg}^{2+}]_o$, did not change the magnitude of facilitation. We explored whether this observation could be attributed to the buffer saturation model of facilitation, since increasing the Ca^{2+} channel-vesicle separation could potentially enhance the role of endogenous buffers.

The characteristics of facilitation in synapses treated with ω -Aga IVA were probed with broad action potentials in the presence of K^+ channel blockers. After Ca^{2+} channel-vesicle separation was increased by ω -Aga IVA, facilitation probed with broad action potentials was still decreased by EGTA injection and increased by 40 mM $[\text{Mg}^{2+}]_o$. EGTA-AM perfusion was used to test the impact of a range of EGTA concentrations in ω -Aga IVA poisoned preparations. Results showed a concentration dependent decrease in facilitation as EGTA concentration rose. Thus, probing facilitation with EGTA and reduced Ca^{2+} influx showed that characteristics of facilitation did not change when the role of endogenous buffers was enhanced by increasing Ca^{2+} channel-vesicle separation. There was no clear indication that buffer saturation had become the dominant mechanism for facilitation after ω -Aga IVA poisoning.

Finally, we sought a correlation between residual Ca^{2+} and the magnitude of facilitation. Using the fluorescence transients of a low affinity Ca^{2+} indicator, we calculated the ratio of fluorescence amplitude measured immediately before a test pulse (Residual Ca^{2+}) to that evoked during an action potential (local Ca^{2+}). This ratio provides an estimate of relative changes between residual Ca^{2+} and local Ca^{2+} important for release. There was a significant increase in facilitation and the ratio when Ca^{2+} influx was reduced by 40 mM $[\text{Mg}^{2+}]_o$. Furthermore, increases in the ratio resulted in an increase in the magnitude of facilitation regardless of the distance between Ca^{2+} channels and releasable vesicles. This correlation suggests the importance of relative changes in residual and local Ca^{2+} to facilitation and lends support to the residual Ca^{2+} hypothesis.

Keywords

Crayfish; Facilitation; Residual Ca^{2+} hypothesis; Buffer Saturation Model

Corresponding author: Contact: Dr. Jen-Wei Lin, 24 Cummington Street, Boston, MA 02215. Phone: 617-353-3443; Fax: 617-353-6340, e-mail: jenwelin@bu.edu.

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Introduction

Synaptic facilitation is a commonly observed form of short-term synaptic enhancement, with a duration lasting from tens to hundreds of milliseconds (Fisher et al., 1997, Zucker and Regehr, 2002). Facilitation is typically elicited by a conditioning stimulus consisting of a single or a burst of action potentials (APs) and is tested by an AP delivered after a defined delay following the conditioning stimulus. Two hypotheses have been proposed to account for facilitation: the residual Ca^{2+} hypothesis (RCH) and the buffer saturation model (BSM). The RCH proposes that enhanced release is due to accumulated free Ca^{2+} resulting from the conditioning stimulus, although the specific processes driven by the free Ca^{2+} are undefined (Dodge and Rahamimoff, 1967, Katz and Miledi, 1968, Zucker, 1999, Zucker and Regehr, 2002). The BSM argues that Ca^{2+} influx during the conditioning stimulus progressively saturates endogenous buffers, resulting in reduced buffering of Ca^{2+} influx evoked by the test AP (Neher, 1998, Rozov et al., 2001, Blatow et al., 2003, Felmy and Schneggenburger, 2004). Since both models have been put forward to explain a vast amount of published data on facilitation, they exhibit similar kinetic properties. As a result, distinguishing between RCH and BSM requires subtle and quantitative arguments.

It has been suggested that the distance between Ca^{2+} channels and synaptic vesicles could play an important role in dictating the mechanism of facilitation (Rozov et al., 2001, Felmy et al., 2003, Matveev et al., 2004). It could be argued that characteristics of facilitation predicted by the buffer saturation model would be accentuated when Ca^{2+} channel-synaptic vesicle separation was large since the probability for endogenous buffers to intercept Ca^{2+} ions, before they reach Ca^{2+} sensors on releasable vesicles, should be increased under such conditions. We have previously reported that P-type channels are more closely coupled to synaptic vesicles than non-P-type channels at the crayfish inhibitory NMJ (Allana and Lin, 2004). Therefore, by selectively blocking P-type channels, we are able to explore the impact of varying Ca^{2+} channel-vesicle separation, and consequently the role of endogenous buffers, on facilitation.

EGTA has been used to differentiate the RCH and BSM. Due to its slow Ca^{2+} binding kinetics, EGTA can be used to reduce residual $[\text{Ca}^{2+}]_i$ without significantly affecting the transient $[\text{Ca}^{2+}]_i$ increase responsible for release. In support of the RCH, studies in cerebellum and at the frog neuromuscular junction have shown that reducing residual $[\text{Ca}^{2+}]_i$ with EGTA is accompanied by a decrease in facilitation monotonically (Atluri and Regehr, 1996, Suzuki et al., 2000, Zucker and Regehr, 2002). However, studies at mammalian cortical synapses indicate that the impact of EGTA is only apparent when the buffer is present at high concentration (Rozov et al., 2001, Blatow et al., 2003). This observation was considered as supporting evidence for the BSM because it was assumed that EGTA at low concentration was unable to compete with endogenous buffers (Blatow et al., 2003), or BAPTA (Rozov et al., 2001), within the time frame of facilitation. Thus, the RCH and BSM can be differentiated by EGTA injection, if the injected concentration is taken into account.

An important manipulation often used to probe facilitation is a change in $[\text{Ca}^{2+}]_o$. At synapses extensively dialyzed with BAPTA, raising $[\text{Ca}^{2+}]_o$ increases facilitation (Rozov et al., 2001). This observation was used to illustrate the buffer saturation mediated facilitation, as it was argued that increased $[\text{Ca}^{2+}]_o$ results in a higher degree of BAPTA saturation following the conditioning stimulus, and consequently less of the buffer would be available to absorb incoming Ca^{2+} ions activated by the test AP. Similar Ca^{2+} dependence of facilitation was also reported at mossy fiber synapses and multipolar bursting neurons to pyramidal cell synapses under physiological conditions, suggesting the operation of BSM in native synapses (Blatow et al., 2003). However, numerical simulation has suggested that raising $[\text{Ca}^{2+}]_o$ does not necessarily enhance facilitation in a system where BSM is the main process underlying

facilitation (Matveev et al., 2004). Instead, it was shown that the impact of raising $[Ca^{2+}]_o$ on facilitation depends on the degree of buffer saturation resulting from the conditioning stimulus. The RCH prediction on the $[Ca^{2+}]_o$ dependence of facilitation is also model dependent. Depending the number and affinity of Ca^{2+} sensors underlying facilitation, raising $[Ca^{2+}]_o$ could lead to either an increase or a decrease in facilitation (Parnas *et al.*, 1982, Yamada and Zucker, 1992, Bertram et al., 1996, Tang et al., 2000, Matveev et al., 2002, Matveev et al., 2006). Despite the potential ambiguity in interpreting results derived from reduced Ca^{2+} influx, this manipulation could still be informative--especially in conjunction with EGTA loading results--if its impact on facilitation is altered after Ca^{2+} channel-vesicle separation is increased. In this report, EGTA injection and reduced Ca^{2+} influx are used to probe facilitation in ω -Aga IVA poisoned preparations.

Materials and Methods

Preparation and electrophysiology

Crayfish, *Procambarus clarkii*, were obtained from Carolina Biological (Burlington, NC). Animals were maintained at room temperature, $\sim 22^\circ C$, until use. All experiments were performed at room temperature. The typical size of the animals was 4-6 cm, head to tail. The opener muscle of the first walking leg was used for all experiments. A presynaptic electrode penetrated the inhibitory axon (inhibitor) to record action potentials and pressure inject Ca^{2+} indicator. The AP measuring electrode was 100–300 μm from the terminals on a central muscle at which fluorescence transients were measured. A suction electrode was used to stimulate the inhibitor. Voltage clamp of muscle fibers was obtained with 2 postsynaptic electrodes, 5-15 M Ω with 1.5-3 M KCl, in a single muscle fiber. GeneClamp 500 (Axon Instruments, Foster City, CA) was used to record IPSC, filtered at 2 kHz. Physiological saline contained (in mM): 195 NaCl, 5.4 KCl, 13.5 $CaCl_2$, 2.6 $MgCl_2$, and 10 HEPES, titrated to pH 7.4 by NaOH. The osmolarity of saline with elevated $[Mg^{2+}]_o$ was maintained by removing the appropriate amount of NaCl. Similarly, in saline containing 20 mM tetraethylammonium (TEA) chloride and 1 mM 4-aminopyridine (4-AP), 20 mM NaCl was removed (T4 saline). Some experiments in physiological saline were carried out in the presence of 0.5 mM 4-AP to enhance neurotransmitter release. This treatment lengthened the duration of the AP by ~ 100 μsec . All chemicals in saline were purchased from Sigma (St. Louis, MO). Solutions containing ω -Aga IVA at 1 nM, 5 nM and 10 nM, as well as Mg^{2+} , were circulated through a 5 ml chamber 3-5 times to ensure equilibration and complete washout of control solutions.

Presynaptic voltage clamp was performed with GeneClamp 500. The inhibitor was first injected with Ca^{2+} indicator and then re-penetrated with voltage and current electrodes. The former contained 250 mM K^+ Methansulphonate and 250 mM K^+ HEPES, 40 M Ω . The current electrode contained 1M K^+ Acetate, 20 M Ω . Calcium imaging site and IPSP were recorded from terminals and muscle fibers near the voltage electrodes (Vyshedskiy and Lin, 1997b).

To reduce Ca^{2+} influx through single channels, we chose to raise $[Mg^{2+}]_o$ rather than decrease $[Ca^{2+}]_o$ for two reasons. First, reducing $[Ca^{2+}]_o$ in T4 saline led to spontaneous firing of axons and muscle contraction, presumably due to a reduction in Ca^{2+} screen of surface potential. Second, in physiological saline, raising $[Mg^{2+}]_o$ resulted in a more rapid, consistent and reversible reduction in IPSP amplitude than lowering $[Ca^{2+}]_o$.

Photometric measurement of Calcium transients

The inhibitory axon was penetrated with an electrode containing 2.5–5 mM Magnesium Orange (MgOrg, $K_D=12$ μM for Ca^{2+}), or Magnesium Green (MgGr, $K_d=7$ μM for Ca^{2+}), dissolved in 400 mM K^+ Methansulphonate, with a final resistance of 15–30 M Ω . For experiments using the high affinity dye Calcium Orange (CaOrg, $K_D=185$ nM), the concentration of dye and

K⁺ Methansulphonate in the electrode were 2.5 and 200 mM, respectively. All dyes were pressure injected, until varicosities close to the injection site were clearly visible. Experiments commenced 15-20 min after dye injection stopped, when the fluorescence level had stabilized. Indicator concentration in the axon was estimated by comparing the fluorescence intensity of the injected axon with a micropipette of similar diameter filled with a calibration solution. The calibration solution was prepared by dissolving the dye in a standard buffer with a final [Ca²⁺] of 100 nM (Ca²⁺ Calibration Buffer Kit with 1 mM Mg²⁺, Molecular Probes, C3721). Using this criterion, a typical injection resulted in an intra-axonal indicator concentration of 200-400 μ M, roughly 10 times lower than that in the microelectrode. Given the low affinity of MgOrg for Ca²⁺ (12 μ M), a 300 μ M injected final concentration is equivalent to a binding ratio of 25, <5% of the endogenous binding ratio of this preparation (~600, (Tank *et al.*, 1995)). This estimate was consistent with observation that facilitation magnitude was comparable between native preparations and those injected with MgOrg. Furthermore, use of 40 mM [Mg²⁺]_o did not result in a change in [Mg²⁺]_i detectable by MgOrg (K_d=3.9 mM for Mg²⁺) which would compromise Ca²⁺ transient measurements. In randomly chosen preparations, resting fluorescence decreased gradually over a period of 60 minutes by 4.5±0.4% (n=6) in 40 mM [Mg²⁺]_o, and by 5.2±1.5% (n=6) in ω -Aga IVA treated preparations.

When the effect of EGTA was investigated, 20 mM EGTA was included in the axon electrode, along with 2.5 mM MgOrg and 400 mM K⁺ Methansulphonate. Using pressure injection, we assumed that EGTA was diluted to a similar extent as MgOrg (~10X), thus to a final concentration of ~2-4 mM. Bath delivery of EGTA was achieved by adding 100 mM EGTA-AM/DMSO stock solution, to perfusion saline at a 1/1000 ratio, i.e. final bath concentration ~100 μ M EGTA-AM. Control saline in EGTA-AM experiments also contained 0.1% DMSO.

Photometric measurement of Ca²⁺ transients in this preparation has been described previously (Vyshedskiy and Lin, 2000). Briefly, a photodiode (S5973-01; Hamamatsu, Bridgewater, NJ) was used to record fluorescence transients on an upright microscope (Axioskop; Zeiss, Oberkochen, Germany) with a 40X or 60X water immersion lens. The photocurrent was measured using a single channel head stage, coupled to Geneclamp 500. A 150W Xenon lamp was powered by an Optiquip 1600 power supply with 770 Lamphouse. Illumination was gated by a shutter (Uniblitz; Vinsent Associates) with a typical duration of ~500 ms and repeated at 0.2 to 0.3 Hz. The area of illumination was restricted by an iris diaphragm customized to allow an illumination diameter of 20~50 μ m with a 40X objective, which typically encompassed ~five varicosities on the upper surface of a central muscle fiber. Fluorescence transients are presented as $\Delta F/F = (F(t) - F_{rest})/F_{rest} * 100$, where F_{rest} represents the fluorescence intensity of stained varicosities in the absence of activity. Background fluorescence in unstained regions was not subtracted.

Data Analysis

All traces used are the average of 80-120 traces. Averaged peak IPSC amplitude recorded in T4 saline varied considerably among preparations (43.7±20.1 nA, n=22). Background noise of the voltage clamp was estimated by calculating standard deviations from the 100 ms (2000 points) period preceding an IPSC. In randomly chosen experiments, the standard deviation of background noise was very low (0.13±0.01 nA, n=8), ~0.3% of averaged peak IPSC amplitude.

Part of this report involves measurement of IPSC evoked by APs broadened with 20 mM TEA and 1 mM 4-AP (T4 saline). Measurement of transmitter release and facilitation in T4 saline is different from that in physiological saline. Figure 1 illustrates a typical IPSC (A1) and Ca²⁺ transient (A2) evoked by a pair of broad APs (A3) in T4 saline. Comparison of the first (—, IPSC₁) and second (---, IPSC₂) IPSC shows that facilitation of the postsynaptic response is characterized by a small increase in peak amplitude (1.1±0.01, n=21, p<0.0001), and an earlier rising phase of IPSC₂ (B1). The small increase in IPSC₂ amplitude is attributable to a

near complete depletion of the readily available pool of vesicles by broad APs in T4 saline (Lin and Fu, 2005). The leftward shift in the IPSC rising phase has been shown to be the main manifestation of facilitation evoked by broad APs or presynaptic voltage steps (Vyshedskiy and Lin, 1997a, Vyshedskiy et al., 2000). Specifically, the magnitude of the leftward shift in the IPSC rising phase relaxed with a decay time constant similar to that of facilitation measured in physiological saline. In addition, measuring IPSC at a fixed time point following presynaptic AP yields characteristics of facilitation similar to those measured in physiological saline. In fact, due to the difference in duration of the two APs (B3), and in order that release could be measured before significant depletion occurs, Ca^{2+} influx and release were best measured before the two APs diverged significantly. Typically, Ca^{2+} transients were measured at 2.5 ms ($\text{CaT}_{2.5}$, double headed arrow between B2 and B3) after $\text{Pre}(\text{Max}_{dv/dt})$. $\text{Pre}(\text{Max}_{dv/dt})$ represents the time point at which the derivative of presynaptic AP was maximal. It should be noted that $\text{CaT}_{2.5}$ was only measured from preparations injected with MgOrg. The low Ca^{2+} affinity of this indicator and its fast kinetics provide the best possible approximation of $[\text{Ca}^{2+}]_i$ level during Ca^{2+} influx (Zhao et al., 1996, Sabatini and Regehr, 1998). IPSC amplitude was measured 0.5 ms later to allow for a delay between Ca^{2+} influx and postsynaptic response. Facilitation was measured as the ratio of IPSCs thus measured ($\text{IPSC}_2/\text{IPSC}_1$, Fig. 1B1). The choice of 0.5 ms delay is based on a previous observation that the delay between the presynaptic Ca^{2+} transient and the IPSC, measured with a macropatch simultaneously from the same cluster of varicosities, is ~ 0.7 ms (Vyshedskiy and Lin, 2000).

With a few exceptions, most studies of synaptic facilitation did not monitor presynaptic Ca^{2+} influx, on the assumption that Ca^{2+} influx activated by the test AP was identical to that activated by the control, conditioning AP. This assumption, however, is not always true (Cuttle et al., 1998). In order to verify that IPSC_1 and IPSC_2 , measured 3 ms after $\text{Pre}(\text{Max}_{dv/dt})$, were evoked by the same magnitude of Ca^{2+} influx, we examined the ratio of $\text{CaT}_{2.5}$ evoked by the first (CaT_1) and second (CaT_2) AP of the paired protocol. The ratio ($\text{CaT}_2/\text{CaT}_1$) was 0.96 ± 0.03 ($n=21$) in T4 saline and 1.14 ± 0.15 ($n=8$) in T4 saline containing 40 mM $[\text{Mg}^{2+}]_o$. (Neither of these ratios was significantly different from 1). However, 5 nM ω -Aga IVA caused a slight decrease in the rate at which CaT_2 rose compared with CaT_1 . In order to ensure that IPSC_2 and IPSC_1 were measured in response to a similar Ca^{2+} influx, we shifted the CaT_2 and IPSC_2 measurements to 2.85 ms and 3.35 ms, respectively, such that ratio of CaT_2 (at 2.85 ms) to CaT_1 (at 2.5 ms) was 1.1 ± 0.1 ($n=6$). The facilitation ratio measured in 5 nM ω -Aga IVA was therefore measured as IPSC_2 (at 3.35) to IPSC_1 (at 3 ms).

Synaptic delay, also used to gauge the magnitude of facilitation, was measured from $\text{Pre}(\text{Max}_{dv/dt})$ to the point at which the IPSC crossed the threshold set to 5 standard deviations of background noise. Only those experiments in which the background noise for control and toxin conditions remained the same were used for analysis.

The use of paired broad APs in T4 saline matches the stimulus pattern used to evoke facilitation in physiological saline. In Physiological saline, 10 APs at 100 Hz were used to activate facilitation and a single AP delivered 100 ms later was used to probe facilitation. A previous study has shown that a single broad AP in T4 saline activated approximately 10 times more Ca^{2+} influx than that triggered by a narrow AP in physiological saline (Vyshedskiy and Lin, 2000). Therefore, the magnitudes of facilitation studied in physiological and T4 saline can be considered comparable in terms of the total influx of Ca^{2+} activated by the conditioning stimulus.

Distinction between F1 and F2 components of facilitation

Facilitation at the crayfish NMJ is known to exhibit distinct F1 and F2 components after 1 or 5 conditioning APs (Magleby, 1987, Bittner, 1989, Vyshedskiy and Lin, 1997a). However, we noticed that the fast decay associated with the F1 component, $\tau=15$ -30 ms, was not consistently

identifiable after a conditioning stimulus of 10 APs. This was presumably due to a large accumulation of the F2 component, which would interfere with identification of the F1 component when using curve-fitting procedures. In addition, transmitter release saturation induced by the 10 AP train may also have blunted the magnitude of the F1 component (Zucker, 1974). In T4 saline, a single broad AP depleted the readily releasable pool (Lin and Fu, 2005) and, though also recovering within 100 ms, made it impossible to demonstrate an F1 component. Despite the fact that a clear distinction between F1 and F2 components was not feasible with our protocols, we assume that the facilitation reported here is mainly due to the F2 component, because 100 ms is more than 3 times the decay time constant typical of the F1 component.

Results

We have investigated changes in the magnitude of facilitation at the crayfish inhibitory NMJ by manipulating Ca^{2+} influx with either ω -Aga IVA or 40 mM $[\text{Mg}^{2+}]_o$. ω -Aga IVA at 5 nM blocks ~75% of the P-type Ca^{2+} channels closely coupled to synaptic vesicles, and increases Ca^{2+} channel-synaptic vesicle separation (Allana and Lin, 2004). Raising $[\text{Mg}^{2+}]_o$, on the other hand, reduces single channel current through all Ca^{2+} channels, resulting in a spatially uniform reduction in Ca^{2+} influx (Lansman *et al.*, 1986). These concentrations of ω -Aga IVA and $[\text{Mg}^{2+}]_o$ were chosen for their matched degree of inhibition of total Ca^{2+} influx.

40mM Mg^{2+} increases facilitation, but 5 nM ω -Aga IVA does not

In physiological saline, facilitation was induced using a train of 10 APs at 100 Hz and tested with a single AP 100 ms later. Figure 2A1 shows the typical effect of 5 nM ω -Aga IVA on synaptic transmission, the first IPSP of the train being reduced to 11.3% of control amplitude. To illustrate the toxin's effect on facilitation, the first IPSP of the train in the toxin was scaled to control level (Fig. 2A1, ----). Scaled IPSP 100 ms after the conditioning train showed a similar height to control IPSP, suggesting no change in facilitation (note that the baseline before the test IPSP is slightly higher in the scaled trace). The impact of ω -Aga IVA on facilitation was quantitated as the ratio of facilitation after and before P-type channels were blocked (F_T). Averaged results from 7 preparations showed that, although ω -Aga IVA reduced control IPSP to $11.7 \pm 0.6\%$ of control level, the toxin did not change the magnitude of facilitation (Fig. 2C1). (There is a clear increase in facilitation during the 100 Hz train. This increase presumably reflects the F1 component of facilitation while the facilitation measured 100 ms after the train is within the time window of the F2 component. In addition, release during the train may have approached saturation and masked facilitation. (See Methods.))

In separate preparations, the effect of 5 nM ω -Aga IVA on Ca^{2+} influx was examined using the high affinity indicator Calcium Orange (CaOrg , $K_D = 185$ nM). Ca^{2+} transients were analyzed at two time points. The reduction in Ca^{2+} influx activated by individual APs was monitored at the maximum amplitude of the Ca^{2+} transient evoked by a single AP (Ca_T). The reduction in residual Ca^{2+} was measured during a 5 ms window just prior to the test AP (Ca_{Res} , Fig. 2A2). Similar to results previously reported (Allana and Lin, 2004), 5 nM ω -Aga IVA significantly reduced both Ca_T and Ca_{Res} (···, Fig. 2C2).

Next, 40 mM $[\text{Mg}^{2+}]_o$ was used to reduce Ca^{2+} influx through both P- and non-P-type channels. Addition of Mg^{2+} (···, Fig. 2B1) reduced IPSP to $9.9 \pm 1.6\%$ of control levels ($n=8$), statistically indistinguishable from that achieved by 5 nM ω -Aga IVA ($0.2 < p < 0.5$). Ca_T and Ca_{Res} were also reduced to levels similar to those induced by 5 nM ω -Aga IVA (Fig. 2B2 & C2). However, in contrast to the effect of ω -Aga IVA, the magnitude of facilitation recorded in 40 mM $[\text{Mg}^{2+}]_o$ was significantly higher than that measured in control saline (Fig. 2B1, C1).

Although previous studies have shown that EGTA inhibits facilitation at the excitor of the crayfish opener NMJ (Delaney et al., 1991, Tang et al., 2000), we document here the effect of EGTA on facilitation at the inhibitory junction of this preparation. EGTA, delivered by pressure injection or bath applied EGTA-AM to a final concentration of 2-4 mM, reduced facilitation significantly without affecting control release ($p < 0.001$, Fig. 1C1) (Allana and Lin, 2004). (See Fig. 3D for the efficiency of 2-4 mM EGTA in buffering residual Ca^{2+} .)

The observation that 5 nM ω -Aga IVA and 40 mM Mg^{2+} have different impacts on facilitation, despite a comparable reduction in total Ca^{2+} influx and control IPSP, is puzzling. One possibility is that increased Ca^{2+} channel synaptic vesicle separation, resulting from selectively blocking P-type channels, may enhance the role of endogenous buffers such that buffer saturation becomes the dominant mechanism for facilitation. Determination of whether this selective block of P-type channels shifts the mechanism of facilitation from the RCH to BSM requires assessing the impacts of 40 mM Mg^{2+} or EGTA on facilitation in ω -Aga IVA treated preparations. Such an analysis is impractical with IPSPs recorded in physiological saline, since ω -Aga IVA has already reduced release to 11.7% of control levels (Table 1). Therefore, we carried out the necessary experiments in T4 saline instead. (See Methods and Figure 1 for details of data quantitation.)

Facilitation probed with high $[\text{Mg}^{2+}]_o$ and EGTA in T4 saline

With APs broadened by T4 saline, we first investigated whether the effects of 40 mM $[\text{Mg}^{2+}]_o$ and EGTA injection on facilitation were similar to those probed with narrow APs. Addition of 40 mM $[\text{Mg}^{2+}]_o$ inhibited release and reduced both $\text{CaT}_{2.5}$ and Ca_{Res} (Fig. 3B (---), see Table 1 for averaged results). To graphically illustrate the effect of high $[\text{Mg}^{2+}]_o$ on facilitation, we first superimposed IPSC_1 (—) with IPSC_2 (---). The superimposed traces recorded in high $[\text{Mg}^{2+}]_o$ were then scaled such that IPSC_1 amplitude, measured 3 ms after $\text{Pre}(\text{Max}_{dv/dt})$, was the same height as that recorded in control T4 saline (Fig. 3A open arrowhead). Scaled IPSC_2 recorded in high $[\text{Mg}^{2+}]_o$ was larger than that recorded in control T4 saline (cross bars on IPSC_2 s), indicating an increase in facilitation. (See Supplementary Figure 1 for superimposition of the 4 IPSCs.) The inset in Fig. 3A shows the averaged and statistically significant increase in F_r induced by high $[\text{Mg}^{2+}]_o$. The magnitude of the F_r increase induced by 40 mM $[\text{Mg}^{2+}]_o$ in T4 saline was statistically indistinguishable from that measured in physiological saline ($0.05 < p < 0.1$).

When EGTA was co-injected with Magnesium Orange to a final concentration of 2-4 mM, Ca^{2+} transients were reduced in amplitude and decayed rapidly to baseline within 30 ms after the AP (--- Fig. 3D), confirming that EGTA effectively reduced residual Ca^{2+} . (Note that the reduction in peak amplitude was due to the prolonged AP duration in T4 saline. Similar injection of EGTA did not change the peak amplitude of MgOrg transients evoked by narrow APs). Similar to its effect with narrow APs, EGTA injection led to a significant reduction in facilitation in preparations perfused with T4 saline (Fig. 3C and inset). Thus, the effect of 40 mM $[\text{Mg}^{2+}]_o$ and EGTA injection on facilitation is similar for both physiological and T4 saline.

Facilitation in the presence of ω -Aga IVA

We next evaluated the impact of ω -Aga IVA on facilitation measured in T4 saline. Addition of 5 nM ω -Aga IVA reduced IPSC amplitude (Fig. 4A1) and $\text{CaT}_{2.5}$ (Fig. 4A2, see also Table 1). However, because 5 nM ω -Aga IVA increased AP duration substantially (Fig. 4A3), neither the peak Ca^{2+} transient ($99.2 \pm 5.0\%$ of control, $n=6$) nor residual Ca^{2+} ($\downarrow$, Fig. 4A2, Table 1) changed significantly. (The large increase in AP duration induced by ω -Aga IVA presumably reflects a close coupling between P-type Ca^{2+} channels and Ca^{2+} activated Cl^- channels.) Following the scaling procedure used in Figure 3, facilitation in 5 nM ω -Aga IVA was significantly greater than in control ($p < 0.0001$, Fig. 4B, C) or 40 mM $[\text{Mg}^{2+}]_o$ ($p < 0.01$, Fig.

4C). Therefore, 5 nM ω -Aga IVA increases facilitation in T4 saline but not in physiological saline. Although the prolongation of AP duration in ω -Aga IVA complicated confirmation of the toxin's effect on facilitation in physiological saline, our original rationale remains valid, namely that we can use 40 mM $[Mg^{2+}]_o$ and EGTA to determine whether characteristics of facilitation have changed after Ca^{2+} channel-vesicle separation has been increased by ω -Aga IVA.

Similar to preparations not treated with ω -Aga IVA (Fig.3), addition of 40 mM Mg^{2+} to synapses pretreated with 5 nM ω -Aga IVA led to an increase in the magnitude of facilitation, by a factor of 1.4 ± 0.1 ($n=5$, $p<0.001$, Fig. 5A). The impact of EGTA in preparations pretreated with 5 nM ω -Aga IVA was also investigated. In this series of studies, differences in facilitation were compared between two separate sets of preparations: those treated with only ω -Aga IVA and those treated with the toxin along with pressure injected EGTA. Since separate preparations were used, F_r , which compares facilitation within individual preparations, cannot be used. Absolute magnitude of facilitation ($IPSC_2/IPSC_1$, F_a) was used instead. Figure 5B shows that EGTA/ ω -Aga IVA co-treated preparations exhibited a facilitation of 2.8 ± 0.1 ($n=6$) which was significantly lower than that in preparations treated with 5 nM ω -Aga IVA alone (3.6 ± 0.2 ($n=16$), $p<0.05$). These results suggest that the impact of high $[Mg^{2+}]_o$ and EGTA injection on facilitation is not altered by increase in Ca^{2+} channel-vesicle separation. (See discussion for considerations of EGTA concentration.)

Facilitation decreases with a gradual rise in intracellular EGTA concentration

EGTA concentration in pressure injection experiment can be inferred from the fluorescence intensity of coinjected Ca^{2+} indicator. However, this approach is cumbersome when examining a range of EGTA concentrations. To achieve finer control of EGTA concentration, we used EGTA-AM in the perfusion saline to gradually increase its intracellular concentration. The level of intracellular EGTA was monitored by changes in Ca^{2+} transients. Furthermore, to avoid complications arising from changes in AP duration between the first and second APs, voltage clamp was used to control presynaptic membrane potential. Experiments were performed in T4 saline with 1 μ M TTX and 10 nM ω -Aga IVA, to block up to 90% of P-type channels (Allana and Lin, 2004). Traces in Figure 6A show a pair of presynaptic steps (A3), Ca^{2+} transients (A2) and IPSPs (A1). The stimulus protocol is similar to that employed for broad APs, 20 ms in duration and 80 mV in amplitude and with an inter-pulse interval of 100 ms. Superimposed traces represent control (—), 18 minutes (---) and 38 minutes (- - -) after EGTA-AM (100 μ M) was introduced. The progression of EGTA penetration is indicated by a gradual reduction in the amplitude, and acceleration in the decay, of Ca^{2+} transients. Panels in Figure 6B compare IPSPs recorded during the first (—) and second (- - -) voltage steps at the indicated time points after EGTA-AM introduction. The leftward shift of $IPSP_2$ becomes smaller as EGTA penetration progresses. The gradual decline in facilitation was calculated by taking the IPSP amplitude ratio at 12.5 ms after the onset of the presynaptic voltage step (Fig. 6B1 arrow). The progression of EGTA penetration was quantitated by measuring the amplitude of the Ca^{2+} transient during a 5 ms period before the onset of the second presynaptic step (Fig. 6A2 double arrow heads), identical to the Ca_{Res} measurement used in the paired broadened AP protocol. The decline in the magnitudes of facilitation and Ca_{Res} progressed in parallel, becoming apparent 10 minutes after the onset of EGTA-AM perfusion and taking an additional 20 minutes to reach the minimal steady state, Fig. 6C. Therefore, qualitatively speaking, EGTA can “out-compete” endogenous buffers and effectively inhibit facilitation starting at a low concentration in preparations in which Ca^{2+} channel-vesicle distance has already been increased by ω -Aga IVA.

To compile EGTA-AM perfusion experiments from different preparations, the magnitude of facilitation and residual Ca^{2+} level were normalized and replotted against each other.

Summarized results from 4 preparations are shown in Fig. 6D as different symbols. Numerical simulation was used to map EGTA concentrations onto the normalized plot in Fig. 6D. (See Supplementary Figure 2 for details of numerical simulation and for the script used in the simulation.) The simulation, based on previously published data from this preparation (Tang et al., 2000, Matveev et al., 2002, Lin et al., 2005), estimates the presence of 0.1 mM EGTA when Ca_{Res} is at 80% of control level. Due to scattering of data points, it is clear that the actual reduction in facilitation at 0.1 mM EGTA is not easy to resolve. The decline in the magnitude of facilitation becomes steeper as EGTA reaches the 0.5 mM range. Thus, although the overall trend indicates a continuous decline in facilitation as EGTA concentration rises, detection of reduced facilitation may not be possible until EGTA concentration is significantly above 0.1 mM.

Correlation between $Ca_{Res}/CaT_{2.5}$ ratio and the magnitude of facilitation

One possible interpretation for the enhanced facilitation associated with reduced Ca^{2+} influx is that the fractional decrease in residual free Ca^{2+} is less than that of the local $[Ca^{2+}]_i$ responsible for release. Here, we explore this rationale quantitatively. Changes in residual Ca^{2+} can be approximated by Ca_{Res} measured with MgOrg, which was used in all experiments performed in T4 saline. For estimates of local $[Ca^{2+}]_i$, which has not been measured to date at the crayfish inhibitor, $CaT_{2.5}$ was chosen because it was the reference point for Ca^{2+} transient and IPSC measurements in T4 saline. With increasing $[Mg^{2+}]_o$, an increase in $Ca_{Res}/CaT_{2.5}$ resulted because the fractional reduction in Ca_{Res} was less than that of $CaT_{2.5}$ in high $[Mg^{2+}]_o$, see traces in Figure 3B for example. Figure 7A shows a positive correlation between F_a and $Ca_{Res}/CaT_{2.5}$ as $[Mg^{2+}]_o$ was increased from control to 40 mM (open symbols). The slower decline of Ca_{Res} in high $[Mg^{2+}]_o$ was also supported by a comparison of the decay time constants of Ca^{2+} transients following the conditioning AP, where the decay time constant increased from 44.7 ± 0.45 in control T4 saline to 52.9 ± 0.6 ms ($n=8$; $P<0.05$) in 40 mM $[Mg^{2+}]_o$. (See Figure 3B for an example of curve fitting.)

It was also possible that $Ca_{Res}/CaT_{2.5}$ might increase if $CaT_{2.5}$ or Ca_{Res} were changed independently. This was evident when comparing facilitation in 5 nM ω -Aga IVA and 40 mM Mg^{2+} treated preparations. The longer AP duration induced by ω -Aga IVA resulted in a Ca_{Res} higher than that measured in 40 mM $[Mg^{2+}]_o$. Because the fractional block of $CaT_{2.5}$ was similar in 5 nM ω -Aga IVA and 40 mM $[Mg^{2+}]_o$ (Table 1), the higher Ca_{Res} in 5 nM ω -Aga IVA resulted in a greater $Ca_{Res}/CaT_{2.5}$ ratio. This higher ratio was accompanied by a larger facilitation in 5 nM ω -Aga IVA as compared to 40 mM Mg^{2+} (Fig. 7A, filled square and Fig. 4C). The increase in the $Ca_{Res}/CaT_{2.5}$ ratio between 5 nM and 10 nM ω -Aga IVA had a different cause. Specifically, though residual Ca^{2+} was similar under these two conditions, $CaT_{2.5}$ was lower in 10 nM than 5 nM ω -Aga IVA. This resulted in a further elevation of both the $Ca_{Res}/CaT_{2.5}$ ratio and facilitation. The same scenario also occurred before and after 5 nM ω -Aga IVA application in T4 saline. Pooling data from Mg^{2+} and ω -Aga IVA experiments revealed a coherent trend, suggesting that the magnitude of facilitation correlates closely with the $Ca_{Res}/CaT_{2.5}$ ratio regardless of the distance between Ca^{2+} channels and synaptic vesicles.

In addition to an increase in test IPSC amplitude, previous studies have shown that, when release is triggered by broad APs, facilitation can be quantitated by a decrease in synaptic delay; namely, stronger facilitation is associated with a larger reduction in the synaptic delay of the facilitated response (Vyshedskiy *et al.*, 2000). We plotted $\Delta Delay_{fac}$, the difference in synaptic delay between IPSC₁ and IPSC₂, against the $Ca_{Res}/CaT_{2.5}$ ratio (Fig. 7B) and found that it exhibited a trend identical to that obtained with magnitude of facilitation (Fig. 7A). (See methods for criteria used in measuring $\Delta Delay_{fac}$.) Since IPSC amplitude and $\Delta Delay_{fac}$ measurements relied on different criteria, the close correlation between $\Delta Delay_{fac}$ and $Ca_{Res}/CaT_{2.5}$ provides further support for the trend derived from IPSC amplitude measurements.

Discussion

Results in this report show that enhancing the role of endogenous buffers by increasing Ca^{2+} channel-vesicle separation at the crayfish inhibitory NMJ does not change the characteristics of facilitation. Specifically, probing synaptic facilitation with EGTA and 40 mM $[\text{Mg}^{2+}]_o$ did not uncover indications that the mechanism underlying facilitation had been shifted to the buffer saturation model. It should be noted, however, that this conclusion is applicable only to protocols used in this report, i.e. where the F2 component of facilitation is evoked by a strong conditioning stimulus of 10 APs at 100Hz or a single broad AP.

In physiological saline, reducing Ca^{2+} influx in a spatially uniform manner with 40 mM $[\text{Mg}^{2+}]_o$ resulted in an increase in F2 facilitation, while suppression of residual Ca^{2+} with EGTA (2-4 mM) led to a reduction in F2. ω -Aga IVA, on the other hand, did not change the magnitude of the facilitation in physiological saline despite a reduction in total Ca^{2+} influx and IPSP amplitude to levels comparable to those recorded in high $[\text{Mg}^{2+}]_o$. This result led us to investigate potential roles for endogenous buffers in facilitation after P-type channels were blocked. In ω -Aga IVA treated preparations, application of 40 mM $[\text{Mg}^{2+}]_o$ increased facilitation while 2-4 mM EGTA reduced facilitation. The impact of EGTA at low concentration (<0.2 mM) was difficult to resolve. Thus, experimental manipulations previously used to test the BSM prove inconclusive in determining whether increased Ca^{2+} channel-vesicle separation results in facilitation mediated by the buffer saturation process. Finally, when the magnitude of facilitation, or changes in synaptic delay associated with facilitation ($\Delta\text{Delay}_{\text{fac}}$), were plotted against the $\text{Ca}_{\text{Res}}/\text{CaT}_{2.5}$ ratio, data points fell on the same regression line regardless of whether Ca^{2+} influx was reduced by $[\text{Mg}^{2+}]_o$ or by ω -Aga IVA. This strong correlation suggests that $\text{Ca}_{\text{Res}}/\text{CaT}_{2.5}$, and by inference residual Ca^{2+} , is crucial in dictating facilitation at this synapse.

The use of high $[\text{Mg}^{2+}]_o$ to reduce Ca^{2+} influx

One of the main assumptions in this study is that raising Mg^{2+} reduces Ca^{2+} influx in a spatially uniform manner. This assumption is based on a previous study showing that Mg^{2+} and other divalent cations reduce Ca^{2+} influx by a flickering block mechanism (Lansman *et al.*, 1986). Although a similar single channel recording has not been performed at the crayfish NMJ, this assumption is based on current understanding that divalent blockers of Ca^{2+} channels interact with the Ca^{2+} selectivity filter which has characteristics shared by all voltage dependent Ca^{2+} channels (Carbone *et al.*, 1997, McCleskey, 1999). Another potential complication is that high $[\text{Mg}^{2+}]_o$ can cause a rightward shift in the voltage dependence of voltage gated channels by surface screening (Hille, 1992). However, the surface screening effect appears to be minimal here for two reasons. First, the amplitude and rising phase of action potentials were only slightly reduced by 40 mM $[\text{Mg}^{2+}]_o$ (Allana and Lin, 2004). These small changes could be easily accounted for by the reduction in NaCl (60 mM) needed to maintain osmolarity. Second, Mg^{2+} was added without removing Ca^{2+} and the surface charge should remain screened by Ca^{2+} (13.5 mM), which has a higher affinity for the membrane surface than Mg^{2+} (Hille *et al.*, 1975). As a result, the kind of large rightward shift, ~ 20 mV, of the Na^+ channel G-V curve induced by high Mg^{2+} and in zero Ca^{2+} would be unlikely to occur here (Hille *et al.*, 1975). Therefore, the assumption that Mg^{2+} administration reduces Ca^{2+} influx in a spatially uniform manner is reasonable.

Despite the fact that 40 mM $[\text{Mg}^{2+}]_o$ did not change background fluorescence monitored with MgOrg ($K_d = 3.9$ mM for Mg^{2+}), it remains possible that a small but undetected increase in resting $[\text{Mg}^{2+}]_i$ did occur. Such a change in resting $[\text{Mg}^{2+}]_i$ could theoretically alter the Ca^{2+} buffering dynamics of endogenous buffers, such as parvalbumin, and lead to reduced buffering of free Ca^{2+} (Lee *et al.*, 2000).

Testing the BSM with 2-4 mM EGTA

Previous studies have taken the concentration dependent inhibition of facilitation by EGTA as supporting evidence for the BSM (Rozov et al., 2001, Blatow et al., 2003). Specifically, buffer saturation was thought to be the mechanism underlying facilitation if EGTA did not alter the paired pulse ratio at low concentrations, but reduced it at higher concentrations. It was reasoned that at low concentrations, due to its slow on-rate, EGTA was unable to compete with fast and high affinity endogenous buffers, whereas raising its concentration compensated for its slow on-rate and thereby enabled EGTA to effectively compete for Ca^{2+} . We used two separate experimental approaches to examine this possibility.

First, the impact of EGTA on facilitation in a ω -Aga IVA poisoned NMJ was tested by pressure-injecting the buffer. Only one concentration of EGTA was examined in this case. Since the concentration of EGTA used here (2-4 mM) is higher than was used in the previous study, 0.2-1 mM (Rozov et al., 2001, Blatow et al., 2003), it could be argued that the 2-4 mM EGTA overwhelmed the endogenous buffers and masked the effects expected according to the BSM. However, 2-4 mM EGTA is not an unreasonably high concentration of EGTA based on two considerations. First, 2-4 mM EGTA was not high enough to affect release probed with narrow APs, even though facilitation was significantly reduced at this concentration (Allana and Lin, 2004). Second, when release was probed with broad APs in T4 saline, IPSCs measured at 3 ms were reduced to $73.3 \pm 3.2\%$ ($n=4$) of control values and this level of block was identical to that achieved in multipolar bursting (MB) cells by 0.2 mM EGTA in the presence of 0.2 mM BAPTA (Blatow *et al.*, 2003). Thus, the effects of 2-4 mM EGTA at the crayfish synapse in T4 saline can be considered functionally equivalent to 0.2 mM EGTA in MB cells. Here we argue that, for the purpose of cross-species comparison, functional equivalency is more relevant than absolute EGTA concentration. Since the details of endogenous buffers in various preparations are unknown, identical EGTA concentration does not necessarily guarantee that Ca^{2+} is buffered to a similar extent in distinct preparations. Therefore, despite the use of different EGTA concentrations, comparison between this and previously published studies is justified on the grounds that the common reference point for the effect of EGTA, namely its inhibition of control release, is matched.

To address the issue of the concentration dependent impact of EGTA on facilitation, we used EGTA-AM to raise the buffer concentration gradually. Although actual EGTA concentrations were not directly measured, Ca^{2+} transients were used to indirectly monitor the progression of EGTA penetration. Both numerical simulation and direct comparison with Ca^{2+} transients obtained from pressure injected EGTA (Fig.3D) suggest that the final intracellular EGTA concentration was about 1-2 mM in EGTA-AM experiments. The timelines of facilitation and Ca^{2+} transients indicate that the magnitude of facilitation decreased as Ca^{2+} transients showed signs of EGTA penetration, Fig.6C. However, due to the scattering of data points summarized from 4 preparations, our results showed some ambiguity. Specifically, the decline in facilitation was shallow at an EGTA concentration of 100 μM and became steep around 500 μM . In other words, it could be argued that this trend supports the BSM because changes in facilitation were too small to be detectable when EGTA concentration was around 100 μM . On the other hand, it is important to note that this concentration-dependent inhibition of facilitation by EGTA need not be grounds for rejecting the RCH. This is because the Ca^{2+} binding site underlying the RCH has been proposed to have a high affinity for Ca^{2+} (Blanton et al., 1989, Yamada and Zucker, 1992, Atluri and Regehr, 1996, Bertram et al., 1996, Tang et al., 2000). Thus, a small reduction in Ca_{Res} may still be sufficient to keep the high affinity site occupied and the magnitude of facilitation unchanged. Therefore, the use of EGTA to discriminate between the RCH and BSM may only be applicable under limited experimental conditions,

The significance of $\text{Ca}_{\text{Res}}/\text{CaT}_{2.5}$ and its implications as regards the RCH

Given the strong correlation between $\text{Ca}_{\text{Res}}/\text{CaT}_{2.5}$ and the magnitude of facilitation, as well as $\Delta\text{Delay}_{\text{fac}}$ (Fig. 5 C,D), we set out to clarify the functional implications of this parameter. Ca_{Res} was estimated from the amplitude of the Ca^{2+} transient just prior to the second AP. Since a low affinity Ca^{2+} indicator was used in all Ca_{Res} estimates, indicator saturation can be considered minimal. In addition, the measurement of changes in Ca_{Res} was compared within individual preparations, minimizing variation between preparations. Thus, changes in Ca_{Res} should represent an accurate approximation of changes in residual Ca^{2+} .

Our imaging technique measures spatially averaged fluorescence signals using a low affinity Ca^{2+} indicator. It has been suggested that the amplitude of fluorescence transients thus measured is proportional to total Ca^{2+} influx (Sabatini and Regehr, 1998). The significance of $\text{Ca}_{\text{Res}}/\text{CaT}_{2.5}$ would be more pertinent if $\text{CaT}_{2.5}$ were proportional to local $[\text{Ca}^{2+}]_i$ at the release site ($\text{Ca}^{2+}_{\text{local}}$). In the case of experiments involving raised $[\text{Mg}^{2+}]_o$, this assumption is not unreasonable because the reduction in total Ca^{2+} influx through flickering block is likely to be proportional to that of single channel current and, therefore, local $[\text{Ca}^{2+}]_i$ (Brandt *et al.*, 2005). In fact, a double logarithm plot of IPSC and $\text{CaT}_{2.5}$ gave rise to a cooperativity of 3.9 (Allana and Lin, 2004), similar to that estimated from uncaging studies (Lando and Zucker, 1994, Bollmann *et al.*, 2000, Schneggenburger and Neher, 2000). This similarity in cooperativity is consistent with the assumption that $\text{CaT}_{2.5}$ and $[\text{Ca}^{2+}]_{\text{local}}$ vary linearly.

A direct relationship between $\text{CaT}_{2.5}$ and local $[\text{Ca}^{2+}]_i$ was less obvious in the presence of ω -Aga IVA, since Ca^{2+} influx would have occurred only through channels not blocked by the toxin. Given the uneven distribution of P- and non-P-type channels, with P-type channels located closer to vesicles, a quantitative co-variation between $\text{CaT}_{2.5}$ and local $[\text{Ca}^{2+}]_i$ may not be immediately obvious. However, it should be noted that IPSC was measured at a sufficiently late time point, 3 ms or more after $\text{Pre}(\text{Max}_{dv/dt})$, that distant channels would also be able to contribute to $\text{Ca}^{2+}_{\text{local}}$ and release, as indicated by the small but significant inhibition of release by EGTA discussed earlier. Therefore, if local Ca^{2+} level is the summation of Ca^{2+} influx from both near and far channels, globally averaged $\text{CaT}_{2.5}$ may not be a bad estimate of locally summated $[\text{Ca}^{2+}]_i$. In fact, synaptic delay exhibited a strong, negative correlation with $\text{CaT}_{2.5}$ as both parameters were changed by increasing the concentration of ω -Aga IVA (Allana and Lin, 2004). This correlation is reminiscent of the correlation between $[\text{Ca}^{2+}]_i$ and synaptic delay observed in uncaging studies (Bollmann *et al.*, 2000, Schneggenburger and Neher, 2000). Therefore, changes in $\text{Ca}_{\text{Res}}/\text{CaT}_{2.5}$ can be considered a reflection of changes in residual Ca^{2+} relative to local $[\text{Ca}^{2+}]_i$ for both high $[\text{Mg}^{2+}]_o$ and ω -Aga IVA treated NMJs, and the strong correlation of this ratio with both the magnitude of facilitation and $\Delta\text{Delay}_{\text{fac}}$ suggests the importance of residual Ca^{2+} in facilitation.

Conclusion

Presynaptic release machinery represents a highly organized structure. Synaptic vesicles, Ca^{2+} channels and proteins around active zones are optimally arranged spatially on a nanometer scale in order to accomplish excitation-secretion coupling at sub-millisecond speed. The functional significance of the spatial organization of active zones has not been fully explored. This report focuses on the question of whether enhancing the role of endogenous buffers by increasing Ca^{2+} channel vesicle separation changes the characteristics of facilitation in a manner suggestive of the buffer saturation model. The results of this approach do not yield a clear-cut answer. The cause of this ambiguity is likely to be multi-faceted. For example, the RCH and BSM are practically indistinguishable kinetically. Unambiguous discrimination between the two models may require knowledge of structural and biochemical details currently not available in this preparation. In addition, more stringent experimental controls than those employed here may also be necessary. Finally, it remains possible that the two mechanisms

coexist and their relative manifestation depends on the degree of endogenous buffers saturation. Further efforts in this or other preparations are needed to answer whether RCH or BSM is the main mechanism of synaptic facilitation under physiological conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

NMJ	neuromuscular junction
BSM	buffer saturation model
RCH	residual calcium hypothesis
AP	action potential
TEA	tetraethylammonium
4-AP	4-aminopyridine
T4 saline	crayfish saline containing 20 mM TEA and 1 mM 4-A
Pre(Maxdv/dt)	the time point at which the slope of presynaptic AP was maximal.
$\Delta\text{Delay}_{\text{fac}}$	the difference in synaptic delay between control and facilitated IPSCs.

Figure 1. Facilitation probed with paired broad APs

A1-3, illustrate IPSCs, Ca^{2+} transients and APs, respectively, activated by a paired of APs in T4 saline. Breaks in the traces indicate location of the stimulation artifact. B, 1-3, Superimposition of conditioning (—) and test (....) IPSCs, Ca^{2+} transients and APs displayed on a faster time scale. Because the second AP was shorter than the first (B3), Ca^{2+} influx was measured before the two APs diverged, i.e. 2.5 ms after $\text{Pre}(\text{Max}_{dv/dt})$, double headed arrow between B2 and B3. IPSC amplitude was measured 0.5 ms later (B1, arrows). Facilitation was measured as $\text{IPSC}_2/\text{IPSC}_1$. The peak amplitude of the second Ca^{2+} transient was smaller than the first, mainly because of the shorter duration of the second AP (....). The Ca^{2+} transient was recorded with MgOrg.

Figure 2. Differential effects of 5 nM ω -Aga IVA and 40 mM $[Mg^{2+}]_o$ on facilitation in physiological saline

Effects of ω -Aga IVA (A) and 40 mM $[Mg^{2+}]_o$ (B) on IPSP (A1,B1) and presynaptic Ca^{2+} transient (A2,B2). Scaling IPSP (----) in the presence of blockers to the same height as that of the first IPSP of the train in physiological saline revealed that while 40 mM $[Mg^{2+}]_o$ substantially increased facilitation, ω -Aga toxin did not. The effects of these blockers on Ca^{2+} influx (A2,B2) were monitored with CaOrg and were obtained from preparations different from those in A1 and B1. Ca_T identifies the maximal amplitude of the Ca^{2+} transient activated by the first isolated AP and is used to monitor the extent of Ca^{2+} influx block. Ca_{Res} measures residual Ca^{2+} during a 5 ms window prior to the test pulse. Traces in A and B share the same time scale. C1: Summary of facilitation, normalized by facilitation in control saline (F_r), in the presence of 5 nM ω -Aga IVA, 40 mM $[Mg^{2+}]_o$ and EGTA. Averaged data in the presence of EGTA include results measured from IPSP and IPSC. C2: Summary of the reduction in Ca^{2+} influx as monitored by Ca_T and Ca_{Res} in 5 nM ω -Aga IVA and 40 mM $[Mg^{2+}]_o$. Due to the small Ca^{2+} influx activated by APs in physiological saline, experiments included in C2 used high affinity indicator CaOrg ($K_D=185$ nM). ** in C1 represents $p < 0.0001$, while * represents $p < 0.001$.

Figure 3. Effects of high $[Mg^{2+}]_o$ and EGTA on facilitation in T4 saline

A, Comparison of facilitation magnitude before and after 40 mM $[Mg^{2+}]_o$. IPSC₁ (—) and IPSC₂ (····) evoked by the paired AP protocol are superimposed to highlight facilitation. To visually illustrate the increase in facilitation induced by high Mg^{2+} , IPSC₁ at 3 ms in 40 mM $[Mg^{2+}]_o$ (right panel in A) was scaled to the same level as that in control saline (open arrowhead and dashed horizontal line). IPSC₂ in high Mg^{2+} exhibited a higher magnitude and facilitation, compare the cross bars in A. Inset, Averaged increase in facilitation in 40 mM $[Mg^{2+}]_o$. B, Ca^{2+} transients measured before (—) and after (····) 40 mM $[Mg^{2+}]_o$. The traces were recorded from the same preparation as that in A. Addition of 40 mM $[Mg^{2+}]_o$ significantly decreased both $Ca_{T2.5}$ and Ca_{Res} . Also note that the reduction in Ca_{Res} is proportionally less than that of the peak amplitude. The slowdown in decay is best illustrated by the single exponential fit, grey lines. The decay time constant of the Ca^{2+} transient was 68 ms in control T4 saline and increased to 78 ms in high $[Mg^{2+}]_o$. The curve fitting range starts 10 ms after the peak of the Ca^{2+} transient and ends 5 ms before the onset of the test AP. C, Facilitation magnitude is decreased by EGTA injection. The impact of EGTA injection on facilitation is illustrated with the same scaling procedure as that show in A. IPSC₂ in EGTA exhibits a reduced amplitude after IPSC₁, in EGTA, has been scaled to the high of control IPSC₁. Inset: Summarized effect of EGTA on facilitation magnitude. D, Ca^{2+} transients averaged from control (—) and EGTA (····) injected preparations. Injection of 2–4 mM EGTA resulted in a decrease in the Ca^{2+} transient during APs, and complete suppression of Ca_{Res} . Control Ca^{2+} transients (n=6) were collected from preparations different from those that received EGTA/MgOrg co-injection (n=4). Gray envelopes surrounding Ca^{2+} transients indicate standard error. The Ca^{2+} indicator used in B and D was MgOrg ($K_D = 12 \mu M$ for Ca^{2+}). B and D share the same time scale.

Figure 4. 5 nM ω -Aga IVA increases facilitation probed with broad APs

A1-3 illustrate IPSCs, Ca^{2+} transients and presynaptic APs, respectively, recorded before (—) and after (····) the addition of 5 nM ω -Aga IVA. Peak amplitudes and Ca_{Res} ($\downarrow$) of Ca^{2+} transients were unchanged in 5 nM ω -Aga IVA because the toxin increased the duration of the presynaptic AP substantially (····, A3). Breaks in the traces indicate the locations of stimulation artifacts. The Ca^{2+} indicator used here was MgOrg. B, IPSCs (IPSC₁, —; IPSC₂, ····) recorded before and after addition of 5 nM ω -Aga IVA. Normalization of IPSC₁ revealed that the magnitude of facilitation was significantly increased by 5 nM ω -Aga IVA. C, Facilitation in the presence of 40 mM $[\text{Mg}^{2+}]_o$ and 5 nM ω -Aga IVA were both significantly higher than control (dashed horizontal line). In addition, the facilitation increase resulting from 5 nM ω -Aga IVA was significantly larger than that resulting from 40 mM $[\text{Mg}^{2+}]_o$.

Figure 5. Effects of high $[Mg^{2+}]_o$ and EGTA on facilitation in preparations pretreated with ω -Aga IVA

A: Averaged results showing that addition of 40 mM $[Mg^{2+}]_o$ significantly increased facilitation even when Ca^{2+} channels-synaptic vesicle distance had been increased by 5 nM ω -Aga IVA. This comparison was performed within the same preparation. B: Summarized results showing that injection of 2-4 mM EGTA in 5 nM ω -Aga IVA treated preparations led to a decrease in facilitation. Since comparisons in B were obtained from different preparations, absolute facilitation (F_a), rather than F_r which was normalized within the same preparations, is used.

Due to the extremely depressed release in double poisoned preparations, ω -Aga IVA+Mg and ω -Aga IVA+EGTA, facilitation calculated in A and B was measured as the ratio of IPSC_2 at 4.85 ms/ IPSC_1 at 4.5 ms. This criterion was also applied to preparations treated with ω -Aga IVA alone to ensure appropriate comparison. IPSC_2 was measured 0.35 ms later than IPSC_1 to accommodate for the slightly slower rise of Ca^{2+} transient during the second AP (see Methods for details).

Figure 6. Facilitation declines during a gradual increase in EGTA concentration in ω -Aga IVA treated preparations

(A) IPSPs (A1) and presynaptic Ca^{2+} transients (A2) evoked by presynaptic voltage steps (A3). The traces represent recordings obtained before (—), 18 (· · ·) and 38 (---) minutes after EGTA-AM perfusion started. (B) IPSPs evoked by the first (—) and second (---) voltage steps are superimposed to illustrate the decline of facilitation from control to 18 and 38 minutes after EGTA-AM perfusion. The IPSPs are expanded from the traces shown in A1. (C) Facilitation and the calcium transients (CaT) declined in parallel during EGTA-AM perfusion. The magnitude of facilitation was measured at 12–12.5 ms after the onset of the presynaptic voltage step, arrow in B1. The Ca^{2+} transients were background subtracted and normalized. Ca^{2+} transients were measured during the 5 ms period before the onset of the second voltage step, double arrows in A2. (D) The magnitudes of facilitation and Ca^{2+} transient shown in (C) were normalized by their respective control values and re-plotted against each other ($\square$). Results from three additional preparations, represented by different symbols, are also included to illustrate the consistency of these results. The continuous line represents the best fit Hill's equation. The Ca^{2+} indicator used in this series was Magnesium Green ($K_D = 7 \mu\text{M}$ for Ca^{2+}).

Figure 7. Magnitudes of facilitation correlate with the ratio $\text{Ca}_{\text{Res}}/\text{CaT}_{2.5}$

The absolute magnitude of facilitation (A) and $\Delta\text{Delay}_{\text{fac}}$ (B) correlated closely with the $\text{Ca}_{\text{Res}}/\text{CaT}_{2.5}$ ratio, implicating residual Ca^{2+} as the main determinant of facilitation magnitude. Data points follow a linear relationship regardless of whether Ca^{2+} influx was reduced by high Mg^{2+} or by ω -Aga IVA. Individual data points were averaged from 6 to 12 preparations. (See text for details of data quantitation.)

Table 1Summary of effects of ω -Aga IVA and high $[Mg^{2+}]_o$ on release and Ca^{2+} influx.

	5 nM ω -Aga IVA (% of control)	40 mM $[Mg^{2+}]_o$ (% of control)
Narrow Aps		
Release	11.7 $\pm$ 0.6(n=7)	9.9 $\pm$ 1.6(n=8)
¹ Ca^{2+} Transient	63.2 $\pm$ 4.5(n=4)	70 $\pm$ 5.2(n=4)
¹ Residual Ca^{2+}	81.1 $\pm$ 5.8(n=4)	79.1 $\pm$ 4.6(n=4)
Broad Aps		
² Release	3.4 $\pm$ 0.4 (n=8)	12.2 $\pm$ 1.4 (n=15)
^{3,4} Ca^{2+} Transient	49.0 $\pm$ 2.9 (n=6)	54.6 $\pm$ 3.4 (n=8)
⁴ Residual Ca^{2+}	113.4 $\pm$ 8.9 (n=6)	59.7 $\pm$ 4.4 (n=8)

¹ Measured with CaOrg as indicator² Measured at 3 ms³ Measured at 2.5 ms⁴ Measured with MgOrg as indicator