A Simple Depletion Model of the Readily Releasable Pool of Synaptic Vesicles Cannot Account for Paired-Pulse Depression

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Abstract

Paired-pulse depression (PPD) is a form of short-term plasticity that plays a central role in processing of synaptic activity, and is manifest as a decrease in the size of the response to the second of two closely timed stimuli. In spite of mounting evidence to the contrary, PPD is still commonly thought to reflect depletion of the pool of synaptic vesicles available for release in response to the second stimulus. Here it is shown that PPD cannot be accounted for by depletion at excitatory synapses made by hippocampal neurons because PPD is unaffected by changes in the fraction of the readily releasable pool (RRP) released by the first of a pair of pulses.

Introduction

In its simplest form, the depletion model of depression proposes that each action potential triggers the release of a constant fraction of the pool of readily releasable vesicles (Liley and North, 1953; Betz, 1970; Zucker and Regehr, 2002). According to this model, when two closely timed stimuli are delivered, the response to the second stimulus will be smaller than the first (depressed) because the RRP is transiently reduced by the number of vesicles released in response to the first stimulus; recovery from depression proceeds as the depleted RRP is refilled over several seconds. Although appealing, this simple model is unlikely to account for the depression that is observed at excitatory hippocampal synapses because back-of-the-envelope calculations incorporating an average release probability per synapse of approximately 0.2 (in 2 mM external calcium; Murthy et al., 1997), and an average RRP size of about 6-10 vesicles (Harris and Sultan, 1995; Schikorski and Stevens, 1997; Murthy et al., 2001), underestimate experimentally observed values of PPD (e.g., Mennerick and Zorumski, 1995; Sippy et al., 2003). Elegant studies at the calyx of Held have implicated heterogeneity of release probability for vesicles within the releasable pool (Wu and Borst, 1999; Sakaba and Neher, 2001), and calcium-dependent inhibition of presynaptic calcium currents (Xu and Wu, 2005) as major mechanisms underlying depression at this giant CNS synapse, but leave open the possibility that depletion could account for depression at smaller hippocampal synapses.
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Here it is shown that simple depletion cannot account for the paired-pulse depression observed at these hippocampal synapses.

Methods

Culture preparation Rat hippocampal neurons isolated from the CA1-CA3 regions were cultured on microislands. Neurons were plated onto a feeder layer of astrocytes that had been laid down 1-7 days earlier and grown in medium containing 10% horse serum. Neurons were grown without mitotic inhibitors and used for recordings after 9 days in culture.

Electrophysiology When a single neuron is grown on a small island of permissive substrate, it forms synapses (“autapses”) onto itself. All experiments were performed on isolated autaptic neurons after AM-EGTA treatment (300 µM for 10 minutes) to buffer intraterminal calcium, thereby removing facilitation. Whole cell voltage-clamp recordings were carried out using Axopatch 200B amplifier (Axon Instruments, Burlingame, CA). The extracellular solution contained (in mM) 119 NaCl, 5 KCl, 1.0 CaCl2, 3.0 MgCl2, 30 glucose, and 20 HEPES. For those experiments where the extracellular calcium concentration was altered, the magnesium concentration was adjusted to maintain a constant concentration of divalent cations. Recording pipettes of 2 to 5 MΩ were filled with 121.5 mM KGluconate, 17.5 mM KCl, 9 mM NaCl, 1 mM MgCl2, 10 mM Hepes, 0.2 mM EGTA, 2 mM MgATP, and 0.5 mM LiGTP. Access resistance was monitored, and only cells with stable access resistance (< 20% deviation from initial value) were included in the data analysis. Data were acquired at a rate of 2 kHz. The membrane potential was held at –60 mV and pairs of EPSCs were evoked by triggering unclamped action currents with 1 ms depolarizing steps separated by a 45 ms interpulse interval. PPD was monitored using the paired-pulse ratio (PPR). To calculate the PPR, the peak amplitude of the response to the second pulse was divided by the peak amplitude of the response to the first pulse. To calculate the PPR at rest for the data in Figure 1, up to 6 PPR values collected at rest were averaged from each cell. To calculate the PPR at rest for the data in Figure 2, the 2 PPR values collected for the 1mM Ca2+ and 2.5 mM Ca2+ trials were averaged from each...
Depletion cannot account for paired-pulse depression cell. For all other data points, the PPR at the indicated time after depletion of the RRP for each cell was calculated from a single pair of responses. A 1 min interval separated the stimulation sets (paired pulses—pool depletion—paired pulses).

Hypertonic solution application The readily releasable pool of vesicles was completely depleted by a 4 - 5 sec application of hypertonic solution (normal extracellular solution without divalent cations plus 500 mM sucrose) using a picospritzer-controlled puffer pipette. A vacuum pipette cleared the hypertonic solution rapidly. The hypertonic solution was applied over the entire island on which the autaptic neuron was located, to ensure that the same population of synapses was activated every time the solution was applied, and that this same population was activated by electrical stimulation at the neuronal cell body.

Results and Discussion

To probe the relationship between depletion and depression, PPD was measured while the size of the RRP was varied. In order to isolate mechanisms underlying depression, experiments were performed using cultured hippocampal neurons that had been treated with the membrane-permeable calcium chelator AM-EGTA to block facilitation; note that fast calcium-dependent processes will not be blocked with this relatively slow chelator. After EGTA-loading, there was no significant effect of RRP depletion on the ratio of excitatory postsynaptic currents (EPSCs) elicited by pairs of electrical stimuli (separated by 45 ms) that were presented at various times after the RRP was emptied by a 5 second-long superfusion of synapses with hypertonic solution: PPD was 0.92 +/- 0.03 at rest (n = 7) and 1.01 +/- 0.13 at 3.7 sec after the end of hypertonic solution application (Figure 1). Could this failure to show altered PPD at depleted synapses be an artifact of the hypertonic solution used to deplete the pool? To examine this question, the experiment was repeated using trains of action potentials (20 Hz for 0.8 sec) to deplete the RRP. Again, there was no significant effect of pool depletion on PPD: PPD at rest was 0.74 +/- 0.08 (n = 7) and 0.77 +/- 0.08 at 3.7 sec after the end of the train (see also Betz, 1970; Bellingham and Walmsley, 1999). Although consistent with the depletion model in its simplest form (i.e. the same fraction of vesicles in the available RRP is
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released by every action potential), these results were somewhat surprising, especially those at the earliest
time points after pool depletion, when one might expect depression to be more pronounced—given that the
RRP should be very small at these times and release of a vesicle would deplete the pool by a greater
fraction than when the pool is larger—raising the possibility that other mechanisms underlie PPD.

To test the depletion hypothesis more directly, PPD was measured under conditions of increased
release probability after depletion of the readily releasable pool. For these experiments, PPD was
compared in the same cell after depletion in the presence or absence of elevated calcium (Figure 2). By
raising calcium from 1 mM to 2.5 mM immediately after application of hypertonic solution, the fraction of the
pool released after depletion could be increased, relative to the fraction released at rest, when the pool was
full. Pairs of stimuli were delivered before and after a 5 sec long application of hypertonic solution to
deplete the RRP in AM-EGTA treated cells. PPD was 0.86 +/- 0.04 (n = 5) at rest in 1 mM Ca$^{2+}$. 6.2 sec
after the RRP was emptied, the amplitude of the first response to the second pair of stimuli was 1/5th the
size of the first response to the first pair (0.20 +/- 0.12) when external Ca$^{2+}$ remained at 1 mM. When
external Ca$^{2+}$ was raised by rapid application of solution containing 2.5 mM Ca$^{2+}$, the amplitude of the first
response to the second pair of stimuli was approximately the same size as the first response evoked by the
first pair (1.00 +/- 0.25). Contrary to the expectations of the depletion model, under these conditions,
where a much larger fraction of the well-depleted readily releasable pool was released because of the
increase in external Ca$^{2+}$, PPD remained identical to that observed at rest in 1 mM Ca$^{2+}$ (0.87 +/- 0.05 after
depletion in 2.5 mM Ca$^{2+}$). Similar results were obtained when trains of action potentials were used to
deplete the pool (data not shown). The lack of effect on PPD after changing external Ca$^{2+}$ was not specific
to synapses with depleted pools, because there was also no difference in PPD at rest when external Ca$^{2+}$
was shifted between 1 mM and 2.5 mM Ca (0.88 +/- 0.06 and 0.83 +/- 0.05, respectively; n = 6). The
insensitivity of PPD to changes in external calcium concentration may seem surprising in light of previous
work (Zucker and Regehr, 2000), but prior studies examining the relationship between the PPR and
external calcium (and release probability in general) have not typically been performed under conditions
that block facilitation; the simplest explanation is that paired-pulse facilitation is sensitive to external
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calcium concentration, but PPD is not. Taken together, these results directly refute the simple depletion
model by showing that even when a significantly greater fraction of the readily releasable pool is released
by the first stimulus of a pair, there is no enhancement of the depression of release in response to the
second stimulus, relative to the amount of depression seen at rest.

Although depletion almost certainly plays a key role in the reduction of response size during a *train*
of action potentials, the data presented here show that a simple pool depletion model cannot account for
PPD at hippocampal excitatory synapses. A number of mechanisms remain plausible candidates for
inducing PPD at this mammalian CNS synapse, including heterogeneity of release probability between
vesicles (Wu and Borst, 1999; Sakaba and Neher, 2001; Moulder and Mennerick, 2005) and use-
dependent inhibition of release machinery (Hsu et al., 1996; Waldeck et al., 2000), but the one most
consistent with the current study is the calcium-dependent inhibition of the presynaptic calcium currents
responsible for triggering release (Xu and Wu, 2005; see also Li et al., 2006), particularly because this
inhibition appears to saturate with small amounts of calcium entry, and would, therefore, be relatively
insensitive to either the presence of a slow calcium buffer, such as the AM-EGTA used here, or changes in
external calcium concentration.

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Figure Legends

**Figure 1.** Paired-pulse depression is not affected by the size of the pool of readily releasable vesicles.

Top trace shows a schematic (not to scale) of the experimental protocol: a pair of brief depolarizing stimuli were delivered 45 msec apart to elicit two synaptic responses at rest (EPSC$_{1a}$ and EPSC$_{2a}$). The first pair of stimuli was followed by a 5 sec application of a Ca$^{2+}$-free external solution made hypertonic by the addition of 0.5 M sucrose to completely deplete the pool of readily releasable vesicles. At varying delays after depletion of the RRP, a second pair of stimuli was delivered to elicit two more synaptic responses (EPSC$_{1b}$ and EPSC$_{2b}$). The lower traces show typical responses to this stimulus paradigm: on the left, the autaptic response at rest, with a PPR of 0.78 in 1 mM external Ca$^{2+}$ after AM-EGTA treatment. The action currents here and elsewhere have been blanked for clarity. At 8.7 sec after the end of the hypertonic solution application, the synaptic responses were reduced to about 1/3$^{rd}$ of their original size, presumably due to depletion of the RRP, but the PPR showed little change (0.81); scale bar: 250 pA, 15 msec. Graph shows pooled results plotting PPR$_b$ (EPSC$_{2b}$/EPSC$_{1b}$) against time after depletion of the RRP by application of hypertonic solution (n = 7) or delivery of a train of action potentials (n = 7); linear regression lines do not deviate significantly from zero.

**Figure 2.** Even when a pair of stimuli releases a large fraction of the readily releasable pool, PPD is not enhanced. The upper left trace in A shows a schematic (not to scale) of the experimental paradigm used for these experiments. First, a pair of stimuli are delivered 45 msec apart to elicit two synaptic responses at rest (EPSC$_{1a}$ and EPSC$_{2a}$), followed by a 5 sec application of hypertonic solution to completely deplete the RRP. At 6.2 sec after depletion of the RRP, a second pair of stimuli is delivered to elicit two more synaptic responses (EPSC$_{1b}$ and EPSC$_{2b}$), either in the same 1 mM external Ca$^{2+}$ in which the first pair of stimuli were elicited, or in elevated (2.5 mM) external Ca$^{2+}$. The trace below the schematic shows the response of an AM-EGTA—treated cell to a pair of stimuli in 1 mM Ca$^{2+}$ at rest. The trace at the upper right shows the response of the same cell to a pair of stimuli in 1 mM Ca$^{2+}$ delivered 6.2 sec after
application of hypertonic solution. The trace at the lower right shows the response to a pair of stimuli in 2.5 mM Ca$^{2+}$ delivered 6.2 sec after application of hypertonic solution. Although the first response to the second pair of stimuli is much smaller in 1 mM Ca$^{2+}$ (EPSC$_{1b}$ << EPSC$_{1a}$), the first response to the second pair of stimuli is about the same size in 2.5 mM Ca$^{2+}$ (EPSC$_{1b}$ ≈ EPSC$_{1a}$). Scale bar: 250 pA, 15 msec. In spite of the large difference in amplitude, there is little difference in the PPR of these responses. Data from 5 cells are plotted in B showing the relative amplitude of the first responses (EPSC$_{1b}$/EPSC$_{1a}$) to the second pair of stimuli in 1 mM and 2.5 mM Ca$^{2+}$. C shows that the PPR in these cells is not altered when a much larger fraction of the readily releasable pool is depleted by the first of a pair of stimuli.
Figure 1. Paired-pulse depression is not affected by the size of the pool of readily releasable vesicles. Top trace shows a schematic (not to scale) of the experimental protocol: a pair of brief depolarizing stimuli were delivered 45 msec apart to elicit two synaptic responses at rest (EPSC1a and EPSC2a). The first pair of stimuli was followed by a 5 sec application of a Ca\(^{2+}\)-free external solution made hypertonic by the addition of 0.5 M sucrose to completely deplete the pool of readily releasable vesicles. At varying delays after depletion of the RRP, a second pair of stimuli was delivered to elicit two more synaptic responses (EPSC1b and EPSC2b). The lower traces show typical responses to this stimulus paradigm: on the left, the autaptic response at rest, with a PPR of 0.78 in 1 mM external Ca\(^{2+}\) after AM-EGTA treatment. The action currents here and elsewhere have been blanked for clarity. At 8.7 sec after the end of the hypertonic solution application, the synaptic responses were reduced to about 1/3rd of their original size, presumably due to depletion of the RRP, but the PPR showed little change (0.81); scale bar: 250 pA, 15 msec. Graph shows pooled results plotting PPR\(_b\) (EPSC2b/EPSC1b) against time after depletion of the RRP by application of hypertonic solution (n = 7) or delivery of a train of action potentials (n = 7); linear regression lines do not deviate significantly from zero.
A

+/- 2.5 mM Ca^{++}

PPR at rest is .75 in 1 mM Ca^{++},
EPSC_{1b}/EPSC_{1a} is .07

At 6.2 sec in 2.5 mM Ca^{++},
PPR is .72

B

![Graph showing EPSC_{1b}/EPSC_{1a} ratios](image)

C

![Graph showing PPR](image)