

# Calcium-Dependent and Calcium-Independent Enhancement of Transmitter Release at the Crayfish Neuromuscular Junction Studied with Fura-2 Imaging

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Although a wealth of experimental evidence exists linking increases in cytoplasmic calcium concentrations to enhancement of evoked transmitter release, quantitative studies that measure and compare the magnitude and temporal characteristics of "residual calcium" to synaptic facilitation are lacking. We have used fluorescent imaging of fura-2 in the intact functioning crayfish neuromuscular junction to address quantitatively the relationship between presynaptic calcium activity and facilitation of action potential-evoked release.

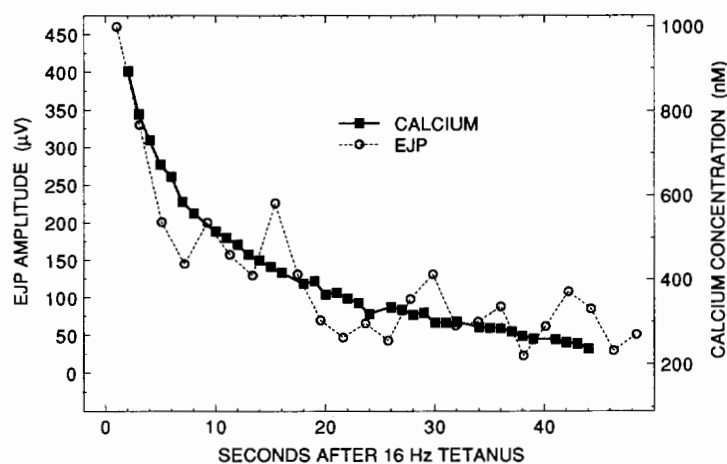
The much-studied neuromuscular junction of the crayfish claw opener muscle is an example of a highly facilitating, nondepressing, tonic synapse. We have further developed this preparation to permit simultaneous measurement of basal calcium concentrations in the presynaptic boutons that release transmitter while recording the postsynaptic responses in a muscle fiber.<sup>1</sup> We have made quantitative comparisons between the decay of calcium accumulations following tetanic stimulation and the decay of tetanically induced facilitation of transmission.

We have concentrated on two of the longer phases of frequency-dependent synaptic enhancement: augmentation, which typically decays with a time-constant of 5–7 seconds, and posttetanic potentiation (PTP), which decays with a time constant that is on the order of a few minutes, depending upon the rate and duration of the tetanus.<sup>2</sup> Starting about 1 s after short tetani, (up to a few hundred action potentials delivered at less than about 30 Hz), augmentation is present, and synaptic enhancement decays with a single exponential time-constant. The calcium accumulation in the presynaptic terminals decays with an identical time-course, indicating a linear correspondence between the residuum of calcium left by the tetanic stimulation and augmentation of release (FIG. 1). This linear relationship appears to persist even with 10-fold or more facilitation of the excitatory junction potential. The persistence of a linear relationship between residual calcium with high levels of facilitation would not be compatible with traditional "power-law" models<sup>3</sup> in which the residual calcium sums directly with calcium influx during the action potential.

With longer stimulation (several thousand action potentials at 20 Hz or greater), a second, longer-lasting phase of facilitation, termed PTP, is produced in addition to augmentation. Thus synaptic enhancement decays with two exponentials. Our measurements demonstrate that calcium accumulation also decays with these same two exponentials. Thus the two time-constants for the decay of calcium accumula-

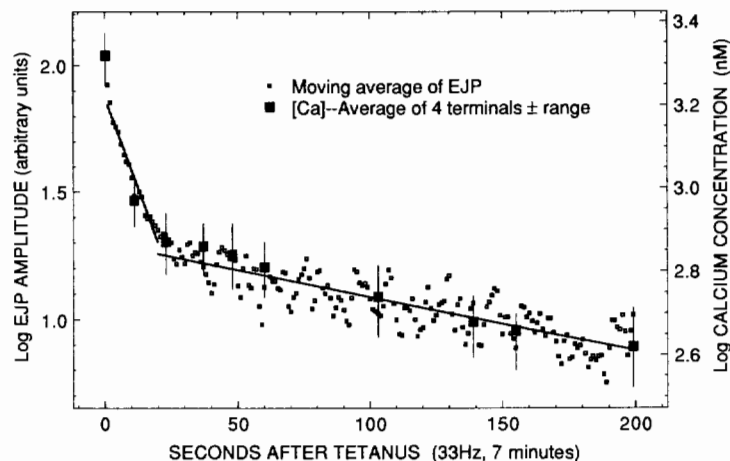
tions are reflected by two time-constants for the slow decay of synaptic facilitation following prolonged tetanic stimulation (FIG. 2).

Addition of exogenous calcium buffer prolongs the buildup of facilitation during tetanic stimulation and prolongs the decay of synaptic enhancement after the offset of an action potential train. In our experiments we use ethylenediamine acetate (EDTA) because its binding kinetics are slow enough that it does not block evoked release even when present at what we estimate to be low millimolar concentrations. The buffer-induced decrease in both the rate of rise and rate of decay of synaptic enhancement is exactly matched by the decrease in the rate of rise of calcium accumulations during tetanic stimulation and the decrease in the rate of recovery of calcium after stimulation. This suggests a causal relationship between the residual calcium and enhancement.



**FIGURE 1.** Decay of augmentation and calcium accumulations after short low-frequency tetanus. Calcium in crayfish claw opener exciter nerve terminals calculated every 1.1 s following the offset of 2 min of 16 Hz facilitation. Time-course of synaptic enhancement following tetanus tested at 0.5 Hz. Results are from a single trial. EDTA iontophoretically injected into the axon to an estimated 1 mM along with fura-2 (about 100  $\mu$ M). Addition of exogenous buffer increased time-constant for both decay of calcium and synaptic facilitation from 6 to 13 s calculated from data greater than 2 s after the offset of 16 Hz stimulation. Calcium and junction potentials scaled to values at pretetanus and about 3 s after tetanus. EJP = excitatory junction potential.

We have also used imaging of calcium in presynaptic terminals to examine whether the facilitatory effects of serotonin<sup>4</sup> at this synapse involve increased basal cytoplasmic calcium concentrations or increased voltage-dependent, action potential-mediated calcium influx.<sup>5</sup> We found little or no increase in the steady-state calcium concentration in presynaptic terminals during a 5-min application of micromolar concentrations of serotonin. An increase of about 50 nM was seen in axonal regions of several preparations, which was reversed quickly after washout whereas enhanced transmitter release persisted for an hour or more. The level of calcium accumulation during 30-s long trains of action potentials was not increased by serotonin. Doubling the extracellular calcium concentration from 50% of normal to 100% of normal



**FIGURE 2.** Stimulation at 33 Hz for 7 min produces an additional long-time constant for the decay of synaptic facilitation corresponding to PTP, which is accompanied by a second slow component of the decay of residual calcium. Solid line is least squared fit to EJP data, illustrating fit by two exponential decay rates. Calcium and EJP scaled to values before tetanic stimulation and approximately 11 s after offset of stimulus. Note semilog coordinates. Adapted from data of Delaney *et al.*<sup>1</sup>.

produced about the same facilitation (4-fold) as serotonin with a clearly detectable 30% increase in accumulation. Broadening the presynaptic action potential with 2–5 mM tetraethylammonium had an even greater effect on calcium accumulations for the same amount of facilitation, indicating that it is unlikely that serotonin acts by broadening the presynaptic action potential. We favor the suggestion that serotonin acts to increase the efficacy of the release apparatus so that normal calcium concentrations result in enhanced transmitter release.

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