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Calcium in Motor Nerve Terminals Associated with Posttetanic Potentiation

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We have used fura-2 fluorescence to study the effects of repetitive stimulation producing posttetanic potentiation (PTP) at crayfish neuromuscular junctions on presynaptic calcium concentration. Fura-2 was injected into the preterminal axon of the excitor motor neuron to the claw opener muscle of a walking leg. Pictures of presynaptic terminals on the muscle surface were obtained with a charge-coupled device camera, ratioed, and converted to spatial images of intracellular calcium concentration. Stimulation of the motor nerve for 7–10 min at 20–33 Hz produced potentiation during the tetanus and PTP following the tetanus. Presynaptic calcium levels in terminal boutons and varicosities rose to about 2 μM during the tetanus and decayed at first rapidly and then slowly back to levels near the initial concentration of about 200 nM. The decay rate of potentiated synaptic transmission was the same as the decay rate of the elevated calcium concentration during the posttetanic period dominated by PTP, when facilitation and augmentation had dissipated. A 13-fold potentiation corresponded to a 500 nM elevation of calcium to about 700 nM. The linear dependence we observed is not consistent with the power law formulation of a residual calcium hypothesis for PTP. During the tetanus, the enhancement of synaptic transmission due to facilitation, augmentation, and potentiation exceeded that expected from the correspondence between PTP and posttetanic calcium levels. This may occur because during the tetanus there is insufficient time for calcium to equilibrate spatially between action potentials, and the submembrane calcium will be higher than the volume-average calcium levels that we detect. Following low-frequency trains (typically 8 Hz for about 35 sec), enhanced synaptic transmission and elevated presynaptic calcium decayed rapidly, within a few seconds. Short high-frequency trains (50–100 Hz for 1–2 min) elicited an additional hours-long elevation of presynaptic calcium, corresponding to, and perhaps responsible for, part of the long-term potentiation of transmission that such stimulation produces at this synapse.

Persistent frequency-dependent enhancement of the excitatory junction potential (EJP) of the crayfish claw opener muscle is a robust and much-studied phenomenon. Stimulation of the excitor axon for a few seconds at frequencies greater than a few hertz produces a short-term enhancement of EJP amplitude resulting from an increased release of transmitter from the presynaptic terminal termed *facilitation.* Stimulation of the axon at higher frequencies, typically between 10 and 30 Hz, for longer periods, 5–20 minutes, so as to deliver several thousand action potentials to the presynaptic terminals, results in enhancement of the EJP, which is resolvable into at least 2 much longer lasting components based on the rate of decay of the enhancement (Wojtowicz and Atwood, 1985). Nomenclature differences exist for describing the different phases of decay of the EJP following tetanic stimulation, but for the purposes of this paper we refer to EJP enhancement, which decays with a time constant of several minutes as *posttetanic potentiation* (PTP), and enhancement, which decays with a time constant of tens of minutes as *long-term facilitation* (LTF). These phases correspond to long-term facilitation and the long-lasting phase of long-term facilitation of Wojtowicz and Atwood (1988). An increase in resting calcium in excitatory presynaptic terminals has been suggested to either wholly or partially account for both short-term facilitation (lasting less than a second) as well as longer-lasting PTP (Atwood and Wojtowicz, 1986; Zucker, 1989). Several manipulations that alter intracellular calcium levels have been shown to increase EJP size (Alnaes and Rahamimoff, 1975; Rahamimoff et al., 1978; Charlton et al., 1982). However, direct measurements of intraterminal calcium concentrations following high-frequency stimulation have not previously been possible because the small size of terminals, typically 2–10 μm, makes them unsuitable for measurements based on absorbance changes such as those obtained with Arsenazo III.

We have used fluorescence ratio imaging of fura-2 signals to measure calcium levels directly within individual presynaptic terminals in a preparation that allows simultaneous recording of EJP amplitudes. We have attempted to relate observed intraterminal calcium levels to enhancement of the EJP directly by applying stimulus regimes to the excitor axon that produces PTP and in some cases LTF while simultaneously imaging $[Ca^{2+}]_i$ in presynaptic terminals. We relate our quantitative measurements of $[Ca^{2+}]_i$ to EJP potentiation within the framework of previous models for facilitation due to residual calcium which were based on theoretical calculations of $[Ca^{2+}]_i$ (Parnas et al., 1982; Zucker and Lara-Estrella, 1983; Fogelson and Zucker, 1985).

Materials and Methods

The distal 2 segments of the first walking leg of crayfish, *Procambarus clarkii,* were secured to the Sylgard-lined bottom of a 60 mm plastic petri dish with cyanoacrylate glue (Tri-Hawk Int., Montreal). This procedure helped reduce movement of the preparation during moderate to

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high-frequency stimulation, which otherwise prevented accurate imaging of calcium. The excitatory axon of crayfish opener muscle of the first walking leg was penetrated near the main "Y" branch in the propodite with single-barrel thin-wall glass microelectrodes filled with 14 or 17 mM fura-2 (pentapotassium salt, Molecular Probes) in 100 mM KCl (50–80 MΩ). Axons were filled with dye by injection of continuous hyperpolarizing current (15–30 nA for 20–50 min). The intracellularly recorded action potential was monitored continuously during injection while stimulating the axon extracellularly at 0.33–0.5 Hz. Passage of 20–30 μC of charge invariably produced sufficient filling of the axon to permit imaging individual terminals within at least 2 mm of the injection site, provided the action potential amplitude remained greater than 60 mV throughout the injection. After filling with fura-2, the injection electrode was gently removed from the axon and the preparation placed on the stage of an upright microscope (Zeiss UEM) fitted with a cooled charge-coupled device (CCD) camera (Photometrics Ltd. Model 220) with computer-controlled data acquisition. The nerve branch in the meropodite containing the excitor axon was placed in a glass suction electrode to permit selective stimulation of the excitor without the inhibitor. The preparation was illuminated with a 1 mm plastic fiberoptic probe positioned with a stage-mounted manipulator. A KCl-filled microelectrode was inserted into a proximal muscle fiber at a shallow angle (approximately 20°) under visual control using a stage-mounted hydraulic manipulator (Narishige, model MO-103M) while viewing the preparation with a 10× objective (Zeiss Neofluor-10). The relative position of terminals and muscle fibers was observed by simultaneously illuminating with 380 nm light through the objective and dim white light from the fiberoptic probe. Terminals that synapsed on proximal muscle fibers were chosen for imaging, and when practical we recorded EJPs from the same muscle fiber upon which the imaged terminal synapsed. When this was not possible we recorded EJPs from adjacent proximal muscle fibers. After penetrating a muscle fiber and obtaining a stable recording of EJPs, the 10× lens was replaced with a 40× Zeiss water-immersion lens to image individual terminals. EJPs were recorded on a Gould pen recorder and EJP amplitudes later measured directly from the chart. Temperature of the preparation was maintained at 15–18°C with a peltier device. Saline (195 mM NaCl, 5.4 mM KCl, 13.5 mM $CaCl_2$, 2.6 mM $MgCl_2$, 10 mM NaHEPES, pH 7.3) was continuously perfused over the preparation at a rate of 1–2 ml/min using a peristaltic pump (Rainin).

Background fluorescence was determined from a part of the muscle on which no terminals were found and subtracted before calculating fluorescence ratios. Autofluorescence of some preparations was appreciable, especially near the exoskeleton, and care was taken to image terminals from areas with the lowest background fluorescence. We were careful to try to match the background to the region that would be imaged for $[Ca^{2+}]_i$ measurements. Possible changes in the background were controlled by remeasuring background at intervals throughout experiments that lasted longer than 15 min.

$[Ca^{2+}]_i$ was determined ratiometrically from signals obtained with 340 and 380 nm illumination. For determination of $[Ca^{2+}]_i$, ratios were measured using square or rectangular masks encompassing 100–225 pixels positioned over the center of a terminal. Calibration of the imaging system was accomplished by measuring fluorescence ratios obtained with 50 μM fura-2 in a cytoplasm-resembling solution (Wallin, 1967) of 17 mM NaCl, 265 mM KCl, and 10 mM KHEPES (pH 7.3), containing 10 mM of either $CaCl_2$ or Na-EGTA. Ratios measured in terminals were converted to calcium concentrations using equation 5 of Grynkiewicz et al. (1985), after application of a viscosity correction corresponding to a 30% reduction in the minimum and maximum 340 nm/380 nm fluorescence ratios (Almers and Neher, 1985; Poenie et al., 1986; Tank et al., 1988). Ratioed images were acquired in 2.5 or 4.5 sec, depending on whether a 1 or 2-sec exposure at each wavelength was used.

We estimated the fura-2 concentration within terminals by comparing the intensity of fluorescence of a 50 μM fura-2 solution excited at 380 nm in a microcuvette of 20 μm pathlength with fluorescence of preterminal axon branches of 5–10 μm diameter. The fura-2 concentration was between 25 and 100 μM in branches leading to presynaptic terminals where measurements of $[Ca^{2+}]_i$ were made for this study.

Repeated measurements of the same terminal during periods of no stimulation or constant 1 Hz stimulation resulted in measured values that varied less than 20 nM over periods of tens of minutes. Similarly, $[Ca^{2+}]_i$ measured in a particular terminal after remeasuring background fluorescence did not typically differ by more than 20 nM. Therefore, we feel that the differences in $[Ca^{2+}]_i$ we measured are likely to be accurate to within at least 30 nM. We are less confident that our measurements of absolute $[Ca^{2+}]_i$ represent true values since a number of variables, including use of an inappropriate area for background subtraction and the use of an arbitrary viscosity correction factor, could contribute substantially to errors in calculating $[Ca^{2+}]_i$. All reported values for $[Ca^{2+}]_i$, including means and SD, are reported to the nearest 10 nM.

Movement during and immediately after tetanic stimulation often prevented $[Ca^{2+}]_i$ measurement based on ratiometric processing of stable superimposed images. In addition to glueing we tried to reduce muscle contraction with glycerol (Gage and Eisenberg, 1967), dantrolene (Desmedt and Hainaut, 1977), ryanodine (Su, 1987), and 2,3-butanedione monoxime (Fryer et al., 1988). However, none of these agents was capable of significantly blocking contraction at concentrations having no effect on synaptic transmission. Because of movement, images of nonsuperimposable terminals at 340 and 380 nM were sometimes observed during and shortly after tetanic stimulation. These were easily identified and were excluded from further analysis.

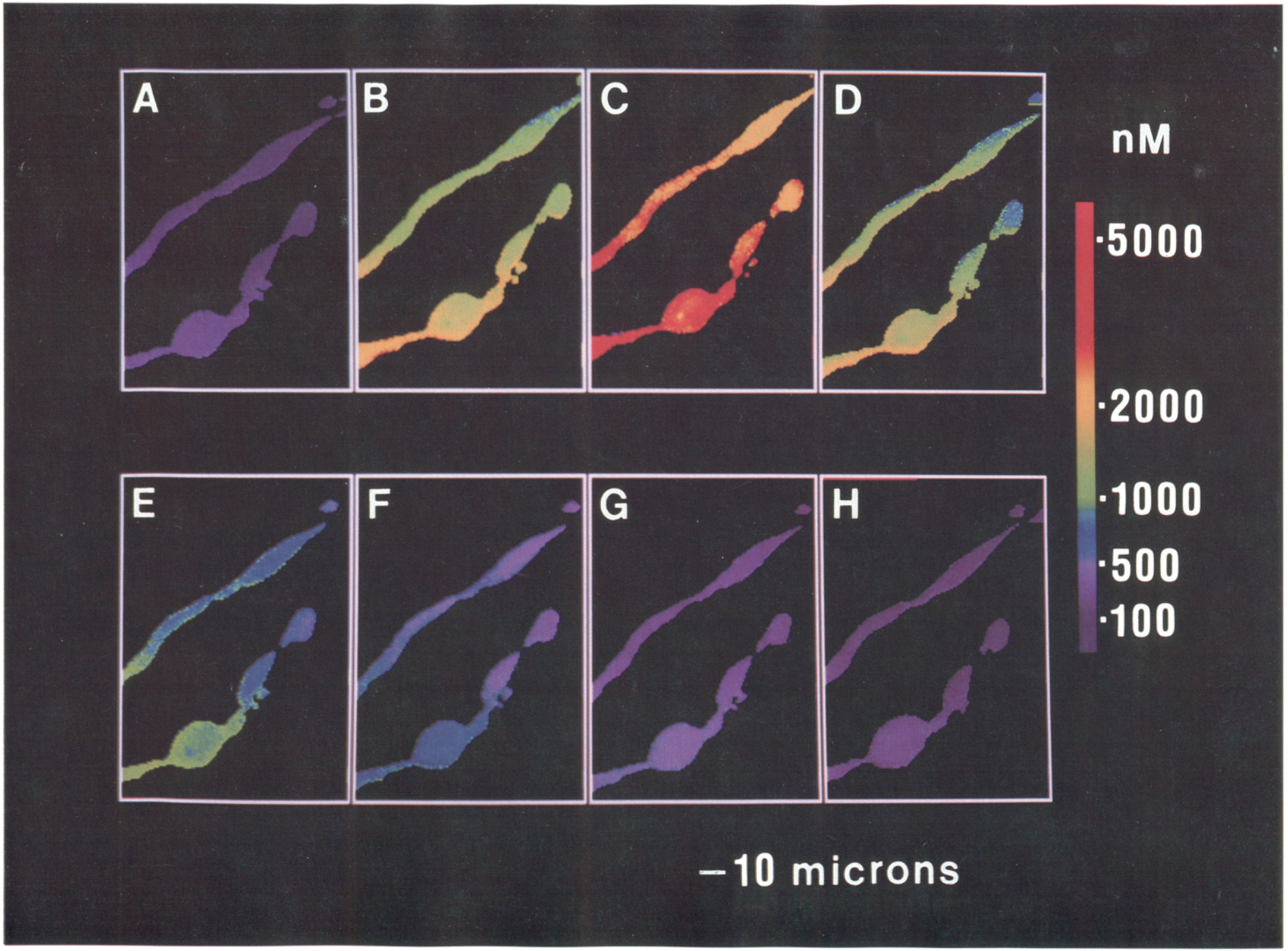
Results

Terminals filled with fura-2

Iontophoretic injection of fura-2 into crayfish motor axons results in a Lucifer yellow-like fill of even the finest terminals and branches. When greater than 30 μC is injected into the axon, many hundreds of synaptic terminals are seen distributed over the surface of the muscle fibers. Fluorescence signals typically remained high enough to image calcium for 6–10 hr after injection. No sequestration of dye within compartments of terminals or axon was observed, and diffusion out the large volume of the axon proximal to the injection site was not a problem. Terminals on the surface of muscle fibers facing the objective were clearly visible as well as terminals located in the spaces between adjacent fibers and on the underside of muscle fibers. The transparency of the muscle fibers forced us to select terminals that did not have filled terminals on the other side of the fiber since the fluorescence from-out-of-focus terminals produced an unacceptable background signal.

In preliminary experiments, some reduction of the EJP amplitude was observed with injections of fura-2 with more than 100 μC of charge (i.e., 3–5 times more than used for this study). Terminals near the injection site in these overfilled preparations had concentrations of fura-2 estimated at 300–500 μM, based on intensity of fluorescence excited at 380 nm. The rate of rise of $[Ca^{2+}]_i$ during short trains and its decline after offset of stimulation were noticeably slower than when 20–30 μC of charge was injected, suggesting that with excessive loading fura-2 could become a significant buffer of calcium within the terminal.

Examining terminals using 380 nm illumination, we routinely observed 2 basic morphological types of terminals. One type was large, approximately 15–20 μm long, often oblong in shape, and occurred more often as swellings or varicosities along the length of fine branches rather than at the ends of branches. A centrally located disk-shaped region with slightly weaker fluorescence was almost always seen in these very large terminals. Terminals located at the ends of branches or on fine collateral branches coming off perpendicular to larger branches tended to be smaller (2–10 μm across) and to be more roundly shaped boutons. These terminals often occurred in grape-like clusters of 3–6 at the ends of branches and did not have a central region of slightly diminished fluorescence. Both types of terminals were used in measuring $[Ca^{2+}]_i$ throughout our experiments, and no consistent differences were observed in this set of experiments.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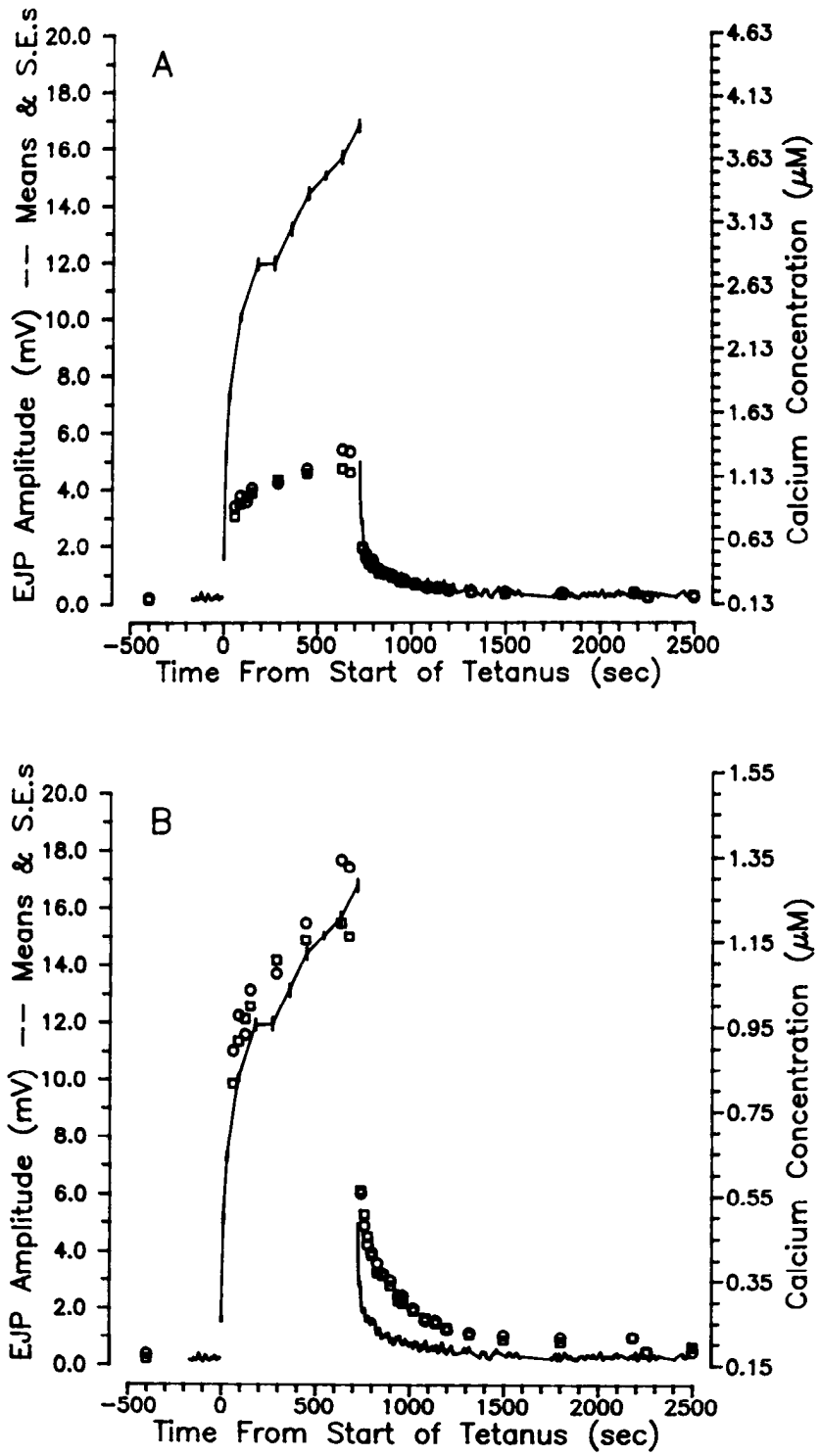

Figure 2. EJP amplitude and calcium concentration measured before, during, and after a 12 min, 20 Hz tetanus. The EJP was recorded from the muscle fiber that was postsynaptic to the presynaptic terminals that were imaged. The symbols show spatially averaged calcium concentration in 2 varicosities. During the pre- and posttetanic period, the *line* represents a running average of 10 successive EJPs. Straight lines are gaps in the recording where EJPs could not be reliably measured. During the tetanus, the *line* connects the averages of 20 EJPs, and the *vertical bars* represent ±1 SEM. *A*, The calcium concentration ordinate scaled so that resting calcium is superimposed on pretetanic EJP amplitude, and posttetanic calcium measurements superimposed on posttetanic EJPs. *B*, Calcium concentration and EJP amplitude scaled to match peak responses during the tetanus. Experiment 3.15.88D.

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Figure 1. Presynaptic free calcium concentration in several excitatory motor neuron terminals on the surface on a muscle fiber in the opener muscle of a crayfish walking leg. Each color image is formed from the ratio of successive fluorescence images to 2 sec exposures at 340 and 380 nm. This ratio is converted to a pseudocolor calcium scale as described in the text. The calcium concentration may be estimated from the calibration scale next to the *color bar*. A 10 μm scale bar is also included. *A*, "Resting" calcium level during 1 Hz stimulation before tetanic stimulation. *B* and *C*, Accumulation of presynaptic calcium at 60 sec and 6 min 36 sec after starting a 7.7 min 30 Hz tetanic stimulation of the motor neuron. The remaining frames record the posttetanic decay of calcium, at 11 sec, 48 sec, 2 min 35 sec, 3 min 47 sec, and 1 hr after the end of the tetanus. Experiment 3.21.88B.

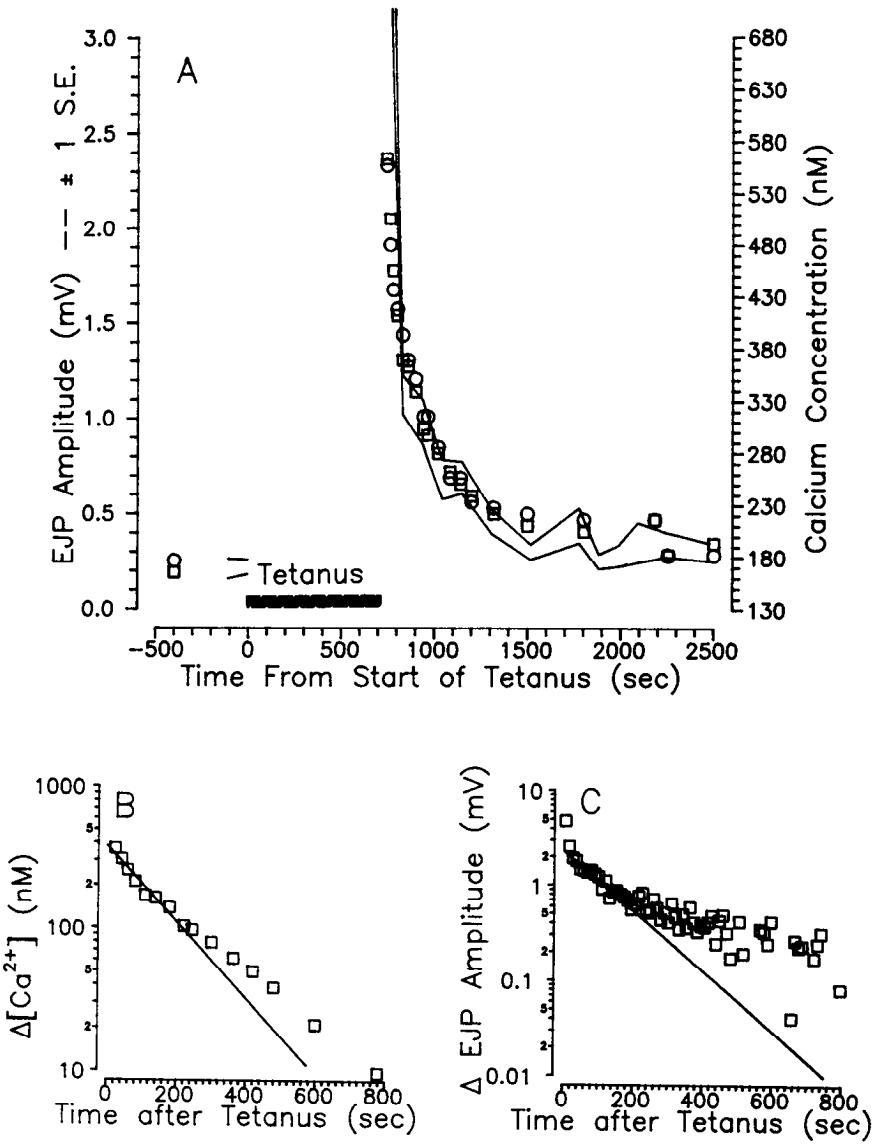

Figure 3. *A*, Posttetanic calcium concentration and EJP data of Figure 2 have been replotted on expanded coordinates to show the similarity of their decay time courses. The 2 lines bound a region encompassing the running average of 10 successive EJPs ±1 SE. A smoothed envelope of ±1 SE was formed by connecting every 100th point. The duration of the tetanus is indicated by the *hatched bar*. *B* and *C*, Logarithm of the increase in EJP and $[Ca^{2+}]_i$ above final values plotted versus time after the tetanus, with a least-square's line fitted to the early posttetanic decays (25–150 sec) as described in the text.

Presynaptic $[Ca^{2+}]_i$ changes during and after long tetani

High-frequency stimulation of the excitor axon was applied to 10 preparations for which dye was successfully injected into excitor axons and stable intracellular recordings from proximal muscle fibers were obtained (3 at 20 Hz for 10 min and 7 at 33 Hz for 7 min). Of these 10 preparations, 7 are included in the analysis of $[Ca^{2+}]_i$ in terminals during PTP. Two of the preparations stimulated at 20 Hz are not included because they did not show robust PTP; EJP size returned to prestimulus levels within 6 min after the onset of stimulation. Data from one of the preparations stimulated at 33 Hz is not included because the signal-to-noise ratio was too low to reliably determine calcium levels in the imaged terminal 10 min after the offset of tetanic stimulation because of a poor injection of dye, although it showed robust PTP with a time course of decay of the EJP similar to other successful preparations. It was often possible to produce a second period of PTP with a second tetanic stimulation after calcium and EJPs returned to normal, but all data included here are from the first application of tetanic stimulation.

Figure 1 shows the ratioed values of $[Ca^{2+}]_i$ obtained during a PTP experiment. Several terminals and interterminal axonal regions are clearly seen. Our spatial resolution of fluorescence measurements was about 1 μm. Threshold values were set sufficiently high to minimize the effects of low fluorescence levels at the very edge of terminals which result from thinning of the tissue, and all measurements of $[Ca^{2+}]_i$ were obtained from pixels located within the center of terminals.

$[Ca^{2+}]_i$ was measured during continuous 1 Hz stimulation since we wished to compare $[Ca^{2+}]_i$ before and after moderate-frequency tetani while sampling EJP amplitudes. When $[Ca^{2+}]_i$ in the same terminal was analyzed both during 1 Hz stimulation and in the absence of any stimulation, the values for $[Ca^{2+}]_i$ measured in 27 terminals from 11 different preparations were a bit higher in all but 2 terminals of one preparation during 1 Hz stimulation: 210 ± 80 nM versus 180 ± 70 nM. Thus, even at 1 Hz, resting $[Ca^{2+}]_i$ is slightly elevated, corresponding to a small EJP facilitation at this frequency (Zucker, 1974).

In those preparations used in the analysis of $[Ca^{2+}]_i$ during tetanic potentiation (7 preparations, 19 terminals, 1–6 terminals/preparation), $[Ca^{2+}]_i$ during 1 Hz stimulation varied be-

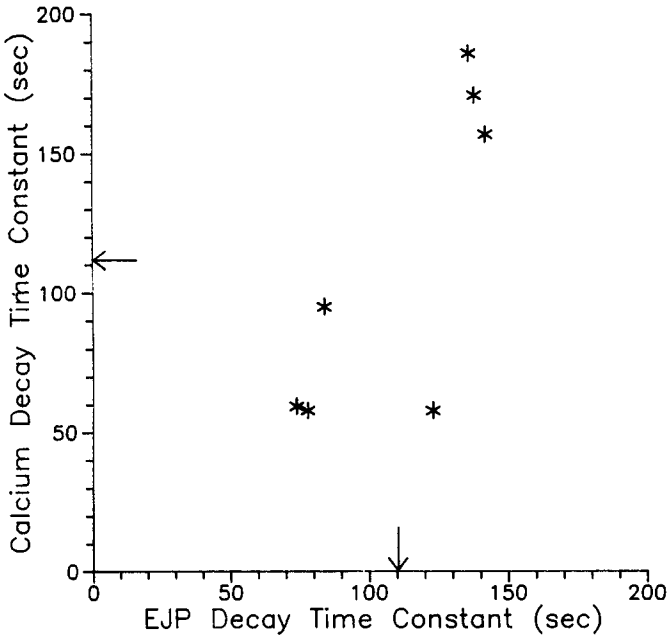

Figure 4. Scatter plot of time constants of exponentials fitted to EJP amplitude and calcium concentration (average of 1–6 terminals) in 7 experiments during the interval of about 25–150 sec after the end of the tetanus. The *arrows* mark the population-average time constants.

tween 140 and 320 nM (mean ± SD: 210 ± 60 nM). Between 25 and 33 min after the end of the tetanus, $[Ca^{2+}]_i$ returned to pretetanus levels: final $[Ca^{2+}]_i$ varied between 110 and 330 nM (mean ± SD: 190 ± 40 nM) measured in 14 terminals from 5 preparations. The last $[Ca^{2+}]_i$ measurements in the remaining 2 preparations were obtained at 8 min in 1 and 13 min in another, and if these values are included then the mean final $[Ca^{2+}]_i$ was 210 ± 40 nM. Ten terminals had final $[Ca^{2+}]_i$ slightly higher than pretetanus, and 9 had $[Ca^{2+}]_i$ slightly lower than pretetanus.

After the offset of tetanic stimulation, the muscle undergoes some relaxation which can continue for up to 30 sec. Therefore, before measuring $[Ca^{2+}]_i$ it was necessary to ensure that the imaged terminals were in focus. The first usable estimate of $[Ca^{2+}]_i$, one in which there was little or no movement during the measurement and for which the terminal was in sharp focus, was usually obtained within 10 sec of the offset of the tetanus. Thereafter, $[Ca^{2+}]_i$ was measured at intervals of 10–20 sec for the next 2–3 min and at longer intervals as the rate of decay of $[Ca^{2+}]_i$ declined.

Posttetanic decay of EJP and $[Ca^{2+}]_i$

Figures 2*A* and 3*A* show that the EJP and $[Ca^{2+}]_i$ appear to decay at similar rates from about 25 sec after the tetanus onward. To more closely compare the rate of decay of $[Ca^{2+}]_i$ with the rate of decay of the EJP following offset of the tetanus, we fitted a single exponential to the data using a least-squares algorithm. We plotted the logarithm of $[Ca^{2+}]_i$ or EJP amplitude, less its final resting value, versus time (Fig. 3, *B, C*) and fitted a straight line to an interval from about 25 to about 150 sec after the tetanus. The exact interval depended on when we happened to take ratiometric images in each experiment. This interval was chosen since it spans approximately 2 time constants for the decay of PTP estimated by other authors (Atwood and Wojtowicz, 1986; Wojtowicz and Atwood, 1988) and starts well after any facilitation of the EJP resulting from augmentation-like processes should have disappeared since these typically have time constants of decay of 5–7 sec in frog (Magleby and Zengel, 1976) and vanish after 30 sec in crayfish (Wojtowicz and Atwood, 1985). We did not attempt to peel off slower components of decay, which appeared in 4 of our 7 preparations. This minor component leads to an overestimation of PTP time constants of 10% or less. Figure 4 shows a plot of τ_{Ca} versus τ_{EJP} from which it is determined that a significant correlation ($\rho = 0.79$, $p < 0.05$) exists between these 2 decay rates. Mean ± SD of τ_{Ca}

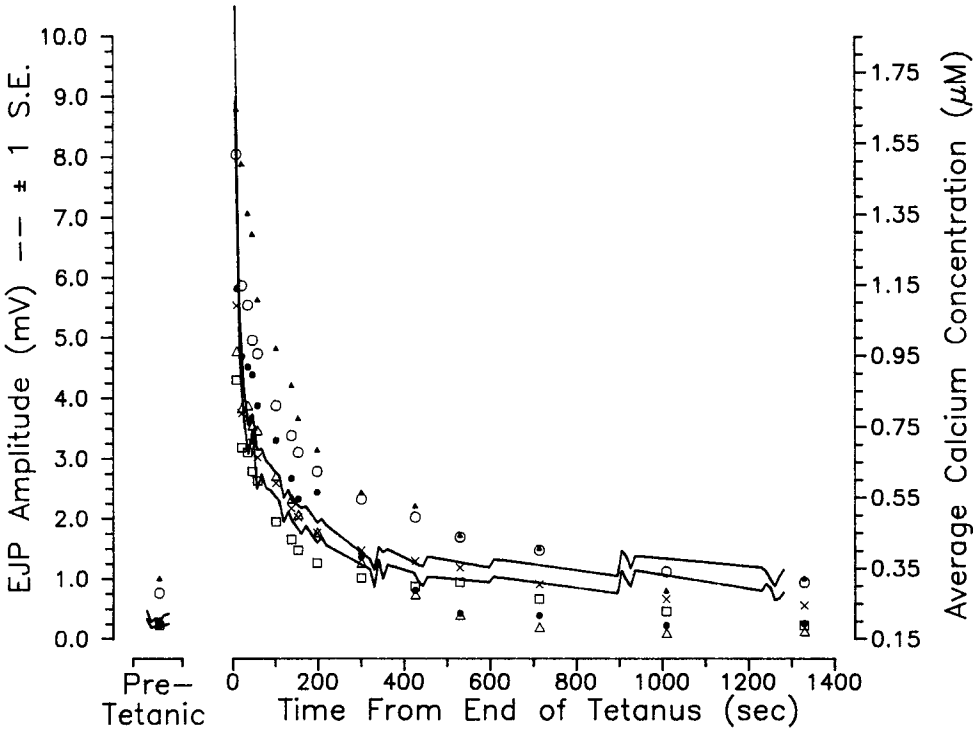

Figure 5. Pretetanic (*unlabeled abscissa*) and posttetanic EJPs and calcium concentrations in an experiment in which calcium was measured in 6 presynaptic varicosities or boutons (each shown as a different symbol). The lines bound a region encompassing ±1 SE of a running average derived from 10 successive EJPs. Experiment 3.21.88B.

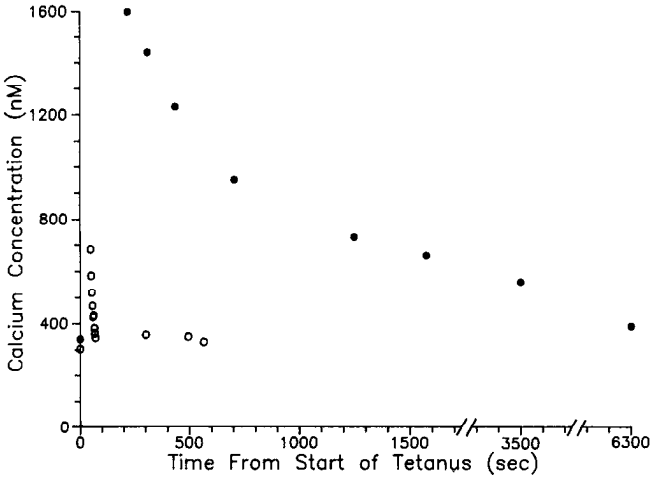

Figure 6. Comparison of $[Ca^{2+}]_i$ after termination of 100 Hz stimulation of the excitor axon for 60 sec (*closed circles*) and 8 Hz stimulation for 45 sec (*open circles*). Prestimulus calcium is measured just before stimulation at $t = 0$. The time constant of decay for calcium following 8 Hz stimulation is 6.6 sec and for 100 Hz is 720 sec during the first 20 min following offset of tetani. The delay of 2.5 min between the offset of 100 Hz stimulation and first $[Ca^{2+}]_i$ measurement was necessary because of continued movement of the preparation during this period. Experiment 12.14.87A.

was 112 ± 31 sec and of τ_{EJP}, 110 ± 57 sec. τ_{Ca} was greater than τ_{EJP} in 4 of 7 cases.

One limitation of our experiments is the inability to measure $[Ca^{2+}]_i$ in all the terminals that synapse on the muscle fiber from which we are recording the EJP. The EJP represents the summed activity of all the synaptic inputs onto the muscle fiber and as such is a measure of the mean synaptic activity. Figure 5 provides some indication of the degree of variability of measured $[Ca^{2+}]_i$ between terminals within the same preparation that is typically observed. In other experiments, fewer terminals were contained within the field of observation, so the probability that the measured terminal(s) accurately represented the mean $[Ca^{2+}]_i$ in the population of terminals synapsing onto the muscle fiber from which the EJP is recorded is obviously reduced.

Calcium and facilitation during the tetanus

When $[Ca^{2+}]_i$ measured 25–150 sec after the end of the tetanus is scaled to the EJP, as shown in Figures 2*A* and 3*B*, then it is clear that $[Ca^{2+}]_i$ measured during the tetanus is not proportional to the EJP amplitude; the EJP level is greater than expected if it were linearly related to $[Ca^{2+}]_i$. The same is true for posttetanic EJPs in the first 15 sec following the end of the tetanus.

During and within 1–2 sec after the tetanus, the increase in EJP amplitude is largely due to the short-term process of synaptic facilitation (Zucker, 1974). In our 7 experiments using long tetani, we observed a maximum facilitation of 27–200 (mean ± SD: 89 ± 58) during the tetanus, while peak $[Ca^{2+}]_i$ during the tetanus varied between 760 and 4.35 μM (mean ± SD: 2.13 ± 0.76 μM). These latter values are a measure of the average calcium through a cross section of the presynaptic terminal. They should therefore underestimate the $[Ca^{2+}]_i$, where it initially enters the terminal, i.e., submembrane $[Ca^{2+}]_i$, if calcium is not evenly distributed in the terminal during moderate-frequency trains of action potentials.

Figure 2 is representative of all preparations in that the $[Ca^{2+}]_i$ measured during the tetanus increased rapidly within a few seconds and then continued to increase more slowly during the tetanus reaching a maximum either at the end of the tetanus or within 2 min of the end. Previous authors (Atwood and Wojtowicz, 1986) have noted that during moderate-frequency trains EJP amplitude typically shows a pattern similar to that in Figure 2, namely, a rapid increase after the onset of stimulation and then a slower maintained increase during the tetanus. In 4 of 6 experiments for which we have reliable measurements of EJPs during the entire train, a slow increase in EJP amplitude was observed during the tetanus in parallel with an increase in average $[Ca^{2+}]_i$. This relationship is clearly seen when peak $[Ca^{2+}]_i$ is scaled to match the peak EJP amplitude during the tetanus as in Figure 2*B*. The other 2 preparations attained their maximum facilitation and maximum $[Ca^{2+}]_i$ within 4 min and thereafter EJP amplitude and $[Ca^{2+}]_i$ did not increase or decreased slightly (<10%) during the rest of the train. Thus, it appears that a slow increase in EJP amplitude during prolonged moderate-frequency stimulation is accompanied by, and is perhaps the result of, a slow increase in average $[Ca^{2+}]_i$.

$[Ca^{2+}]_i$ and PTP magnitudes after the tetanus

In an attempt to relate potentiation of the EJP with $[Ca^{2+}]_i$ in a time-independent manner across experiments, we compared these 2 variables at a point in 6 of 7 preparations where the EJP was potentiated approximately 10-fold. One preparation is not included because we lost our penetration of the muscle fiber during the tetanus and reimpaled a fiber before the offset of tetanic stimulation that may or may not have been the same fiber, so we were unable to confidently compare the pre- and posttetanus EJPs. A roughly 10-fold potentiation was usually seen sometime near the second measurement of $[Ca^{2+}]_i$ after the offset of the tetanus (16–35 sec). $[Ca^{2+}]_i$ was 700 ± 210 nM (560–1050 nM) when EJPs were facilitated an average of 12.6 ± 3.6 times (9.2–18.7 times).

$[Ca^{2+}]_i$ and long-term facilitation

For 4 of the 7 experiments using moderate-frequency trains, stable recordings of EJPs were maintained from the same muscle fiber throughout the experiment and for at least 30 min after the offset of tetanic stimulation. Evidence for a long-lasting phase of facilitation of the EJP (LTF), persisting more than 30 min, was seen in these preparations (34–180% enhancement). Of these preparations showing LTF, 2 had calcium levels that were slightly less than prestimulus baselines (−8 and −11%, e.g., Fig. 5), while in 2 $[Ca^{2+}]_i$ was still elevated (15, 26%: e.g., Fig. 3). In this study, these differences in $[Ca^{2+}]_i$ are too small for us to reliably differentiate from no change at all.

$[Ca^{2+}]_i$ during brief low-frequency trains

We also observed presynaptic calcium changes and EJP facilitation during short low-frequency trains (7–9 Hz for 30–45 sec). In 4 experiments where both variables were recorded, a tetanic facilitation of 11.5 ± 2.2 (mean ± SD) was accompanied by a rise in $[Ca^{2+}]_i$ from 240 ± 70 to 610 ± 190 nM. Recovery of $[Ca^{2+}]_i$ following these trains occurred quickly, with time constants between 5 and 10 sec (Fig. 6). This phase of enhanced synaptic transmission has been called phase I of LTF (Wojtowicz and Atwood, 1985) and resembles the phase termed augmentation at frog neuromuscular junctions (Magleby and Zengel, 1976). The relationships between $[Ca^{2+}]_i$ and facilitation following low-frequency trains was variable. In some instances, $[Ca^{2+}]_i$ decay was linearly related to decay of EJP, while in others, the

EJP declined faster than $[Ca^{2+}]_i$. Undoubtedly part of this variability is the result of the normally highly fluctuating nature of the EJP and the small number of EJPs that can be sampled during the few seconds after the offset of the low-frequency stimulation. The rapid decline of synaptic transmission prohibits EJP averaging. This is not a problem with PTP experiments because potentiation is so long-lasting that large numbers of EJPs can be sampled and averaged without distorting measurement of the time course. Further experiments are required to determine the relationship between τ_{EJP} and τ_{Ca} following low-frequency trains of stimuli.

Short high-frequency trains

Short trains of very high frequency stimulation are reported to produce LTP of the EJP (Baxter et al., 1985). It is difficult to maintain a stable penetration during 50–100 Hz stimulation of the excitor axon so we did not attempt to combine recording EJPs with imaging while stimulating at these high frequencies. In 2 of 2 preparations in which we stimulated at 50 Hz for 2 min and in 2 of 2 preparations in which 100 Hz stimulation was applied for 1 min, $[Ca^{2+}]_i$ remained elevated above prestimulus levels for more than 45 min (Fig. 6). The effects of shorter (e.g., 20 sec) trains at 100 Hz were not examined.

Discussion

We have demonstrated the practicality of simultaneously monitoring calcium concentrations within presynaptic terminal varicosities and boutons while recording postsynaptic EJPs. Our results regarding the magnitude of facilitation during low- and moderate-frequency trains, the time course of the decay of the EJP following tetanic stimulation of the excitor axon, and the dependence of PTP on moderate-frequency stimulation for long periods of time are similar to other studies (e.g., Atwood and Wojtowicz, 1986). This suggests that injection of fura-2 and illumination with short-wavelength light do not alter the normal physiology of the nerve terminal.

Mechanism of PTP

The posttetanic increase in EJP amplitude consists of several phases differentiated primarily on the basis of time course. The first 2 phases, with characteristic decay times of about 20 and 400 msec, are termed the first and second components of synaptic facilitation (Zucker, 1974). A similar synaptic facilitation exists at vertebrate neuromuscular junctions (Mallart and Martin, 1967; Magleby, 1973a) and has been shown to be dependent upon calcium entry during the conditioning stimuli (Katz and Miledi, 1968). Facilitation is usually regarded as a consequence of the nonlinear dependence of transmitter release upon calcium activity—the result of calcium influx from test impulses summing with residual calcium remaining from prior activity (Zucker, 1989).

Following facilitation, a phase of posttetanic increase in transmitter release decaying in tens of seconds has been observed. It is called augmentation at vertebrate neuromuscular junctions (Magleby and Zengel, 1976) and phase I of LTF at crayfish neuromuscular junctions (Wojtowicz and Atwood, 1985). At vertebrate neuromuscular junctions, this phase is also dependent on calcium influx (Weinreich, 1971; Erulkar and Rahamimoff, 1978; Lev-Tov and Rahamimoff, 1980) and is thought to represent a late phase in the removal of residual calcium (Fogelson and Zucker, 1985).

Following augmentation, a phase of increased posttetanic transmitter release lasting several minutes has been termed potentiation, or PTP, at vertebrate neuromuscular junctions (Magleby, 1973b), and phase II of LTF term facilitation at crayfish neuromuscular junctions (Wojtowicz and Atwood, 1985; Atwood and Wojtowicz, 1986). Potentiation seems to be only partly dependent upon calcium influx during the tetanus (Rosenthal, 1969; Lev-Tov and Rahamimoff, 1980). Potentiation has been observed to occur in the absence of calcium influx during conditioning stimuli and to be associated with sodium accumulation during the tetanus (Atwood, 1976; Lev-Tov and Rahamimoff, 1980; Atwood and Wojtowicz, 1986; Wojtowicz and Atwood, 1988). It has often been proposed that this sodium accumulation operates by raising presynaptic calcium, which in turn causes PTP. Although we have not yet proved this hypothesis, we provide here the first direct evidence of a correlation between elevated calcium level at nerve terminals and the presence of PTP. The close correlation we find between the rates of decay of the resting intraterminal calcium and the evoked EJP for several minutes after the offset of stimulation suggests that this elevated resting calcium may be the dominant factor producing enhanced transmitter release during PTP. PTP at central molluscan synapses has also been attributed to presynaptic calcium accumulation, based on a correlation between the decay of PTP and that of intracellular calcium in the soma and major neurites measured with arsenazo III spectrophotometry (Connor et al., 1986) and an additional correlation of PTP with the decay of calcium-activated potassium current recorded in the cell body (Kretz et al., 1982). Our present methods extend the measurement of calcium to the actual terminal boutons at which transmitter release occurs.

Following PTP, a phase of increased transmitter release lasting hours has been observed at crayfish neuromuscular junctions and termed phase III or the long-lasting phase of LTF (Wojtowicz and Atwood, 1985, 1988). This process can be evoked by depolarization in the absence of either calcium or sodium influx, and its mechanism remains obscure. We also observed the late phase of LTF in most of our experiments, but we cannot say with certainty whether it is usually accompanied by a persistent increase in presynaptic calcium level (see below).

In addition to these phases of synaptic enhancement caused by long tetani of moderate frequency, brief high-frequency trains can also elicit LTP of transmitter release lasting for hours (Baxter et al., 1985). Our evidence indicates that this process is also accompanied by a persistent increase in $[Ca^{2+}]_i$ in motor nerve terminals and suggests that at neuromuscular junctions, at least part of LTP may also be a consequence of elevated presynaptic calcium levels.

Quantitative dependence of PTP upon residual calcium

In these experiments, we found a posttetanic increase in evoked transmitter release of about 13-fold to be accompanied by an increase in presynaptic calcium to about 0.70 μM from a resting level of about 0.2 μM. If this residual calcium is the *cause* of PTP, then we may say that an increase in calcium concentration of 0.50 μM is sufficient to cause a 13-fold facilitation. This may be compared with model simulations of synaptic facilitation at frog neuromuscular junctions thought to be caused by residual calcium. Fogelson and Zucker (1985) proposed that a 5-fold facilitation (occurring in response to briefer trains) might be accompanied by about 4 μM of residual calcium.These simulations represented residual calcium remaining from prior influx and contributing to facilitation and augmentation, while in our

experiments, the increase in residual calcium may be a consequence of some effect of sodium accumulation, such as reduction of calcium efflux or even inward calcium leakage by sodium/calcium exchange. Nevertheless, if elevated calcium, whatever its origin, is responsible for facilitation, augmentation, *and* PTP, then a given level of residual calcium should always correspond to a given level of increased transmitter release.

Our observation of a 13-fold potentiation in the face of 0.5–1 μM calcium is substantially greater than the 5-fold facilitation and augmentation accompanying 4 μM calcium predicted in the simulations of Fogelson and Zucker (1985). Moreover, the apparently linear relationship between PTP and residual calcium in the posttetanic period is also surprising. In its simplest form, the residual calcium model predicts that a residual calcium, R, expressed as a fraction of the peak calcium at release sites reached during a single impulse, will potentiate release by a factor $(1 + R)^n$, where n is the calcium cooperativity of transmitter release (Zucker, 1987). For very small R, this reduces to $1 + nR$. Then potentiated release would be proportional to residual calcium, and they would decay with identical time constants. The calcium cooperativity, n, has been estimated to be about 4 (Fogelson and Zucker, 1985), although this is likely to be an underestimate (Parnas et al., 1982; Barton et al., 1983), and a value of 5 may be more appropriate (Zucker and Lara-Estrella, 1983). We have no direct measure of peak calcium during an action potential at submembrane release sites during a spike, so we cannot assign a value to R from our measurement of residual calcium. However, our observation of a 13-fold potentiation about 25 sec after tetani suggests that, for $n = 5$, $R = 0.67$. PTP decays to about 5-fold at about 300 sec after tetani, implying a drop in R to 0.38. Thus, during the period that we find a close correspondence of PTP decay to residual calcium, the above calculation indicates that residual calcium is *not* a small fraction of peak calcium at release sites during a single action potential. The linear approximation to $(1 + R)^n$ is not valid in this range of R, and the results are not consistent with this formulation of PTP arising simply from the nonlinear dependence of transmitter release on presynaptic calcium activity. Only in the first few seconds after a tetanus did EJP amplitude depart significantly from a linear dependence on residual calcium in the direction suggested by the above model.

Another implication of our results is that the simple nonlinear model discussed above implies that the peak calcium concentration reached at release sites during a spike is only about 1 μM. This follows from $R = 0.67$ corresponding to a calcium level of about 700 nM. This magnitude of peak calcium concentration is substantially less than that predicted from models of diffusion of calcium from calcium channels open during an action potential (Fogelson and Zucker, 1985; Simon and Llinás, 1985). This may be another indication of the inadequacy of a simple power law description for the effect of residual calcium on transmitter release.

Several factors might be responsible for these discrepancies. We are relating calcium in a few terminals to EJPs comprising release from many terminals, and our selection of surface terminals may not reflect the behavior of others that we do not sample. The functional form of the relationship between calcium and transmitter release may be rather different from the simple power law equation often assumed. It is also possible that residual calcium acts to increase transmitter release in PTP by other mechanisms than simply summing with calcium entering during test impulses. For example, calcium may act to mobilize vesicles and increase the probability of their attachment to release sites (Llinás et al., 1985).

An increase in calcium sufficient to potentiate EJPs 13-fold should also significantly accelerate the spontaneous release of quanta and increase the miniature EJP (MEJP) frequency. We did not measure MEJP frequency in these experiments, but an earlier study (Zucker and Lara-Estrella, 1983) on this preparation reported a correlation between EJP facilitation and elevation of MEJP frequency consistent with the hypothesis that residual calcium underlies both.

High level of transmission during the tetanus

When we scaled EJP decay to match the decay of $[Ca^{2+}]_i$ 25–30 sec after the offset of 20–33 Hz stimulation, the EJP amplitude during and shortly after the tetanus was larger than indicated by this posttetanic correspondence. One reason for this is that fura-2 fluorescence signals provide a measure of the average $[Ca^{2+}]_i$ across the diameter of the terminal and as such underestimate $[Ca^{2+}]_i$ near the inner surface of the membrane where it is entering the terminal, at least during tetani where the interspike interval is 30–50 msec. We therefore expect that facilitation of the EJP will be greater for a given $[Ca^{2+}]_i$ measured *during* a moderate-frequency train than even a few seconds *after* the offset of stimulation when local calcium domains have collapsed through diffusion (Fogelson and Zucker, 1985; Simon and Llinás, 1985), and calcium is more evenly distributed in the terminal. It is also possible that at high levels of $[Ca^{2+}]_i$, a nonlinear relationship governs the effect of residual calcium on the release of transmitter.

During low-frequency trains (7–9 Hz), an 11.5-fold enhancement of the EJP is associated with a measured $[Ca^{2+}]_i$ of 610 nM, which is similar to the 12.6-fold enhancement with $[Ca^{2+}]_i$ of 700 nM 25 sec after the offset of a moderate-frequency stimulation. This suggests that equilibration of $[Ca^{2+}]_i$ is substantially complete within 100 msec after an action potential, in agreement with our estimate of 90% equilibration in a 5 μm sphere derived from the Smith and Zucker (1980) calcium-diffusion model.

By applying the observed relationship between $[Ca^{2+}]_i$ and 3- to 10-fold PTP, we can estimate how much of an increase in $[Ca^{2+}]_i$ would be required to cause a 50% enhancement of the EJP as is seen during LTF. With resting $[Ca^{2+}]_i$ measured as 200 nM corresponding to no enhancement and 600 nM $[Ca^{2+}]_i$ corresponding to 10-fold enhancement as measured in this study, we calculate that a 20 nM increase in $[Ca^{2+}]_i$ would be sufficient to account for a 1.5-fold increase in EJP. Such a small increase in $[Ca^{2+}]_i$ is too small for us to have detected reliably in this study, especially in view of the long (40–50 min) interval between measurement of the prestimulus $[Ca^{2+}]_i$ and the $[Ca^{2+}]_i$ measured during LTF. Our results do not, therefore, permit any conclusion regarding a contribution of changes in $[Ca^{2+}]_i$ to LTF. The even longer lasting LTP following brief intense tetani was, however, apparently accompanied by an increase in $[Ca^{2+}]_i$.

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