Characterization of postsynaptic Ca\(^{2+}\) signals at the *Drosophila* larval NMJ

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Submitted 18 January 2011; accepted in final form 16 May 2011

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Desai SA, Lnenicka GA. Characterization of postsynaptic Ca\(^{2+}\) signals at the *Drosophila* larval NMJ. *J Neurophysiol* 106: 710–721, 2011. First published May 18, 2011; doi:10.1152/jn.00045.2011.— Postsynaptic intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) has been proposed to play an important role in both synaptic plasticity and synaptic homeostasis. In particular, postsynaptic Ca\(^{2+}\) signals can alter synaptic efficacy by influencing transmitter release, receptor sensitivity, and protein synthesis. We examined the postsynaptic Ca\(^{2+}\) transients at the *Drosophila* larval neuromuscular junction (NMJ) by injecting the muscle fibers with Ca\(^{2+}\) indicators rhod-2 and Oregon Green BAPTA-1 (OGB-1) and then monitoring their increased fluorescence during synaptic activity. We observed discrete postsynaptic Ca\(^{2+}\) transients along the NMJ during single action potentials (APs) and quantal Ca\(^{2+}\) transients produced by spontaneous transmitter release. Most of the evoked Ca\(^{2+}\) transients resulted from the release of one or two quanta of transmitter and occurred largely at synaptic boutons. The magnitude of the Ca\(^{2+}\) signals was correlated with synaptic efficacy; the Is terminals, which produce larger excitatory postsynaptic potentials (EPSPs) and have a greater quantal size than Ib terminals, produced a larger Ca\(^{2+}\) signal per terminal length and larger quantal Ca\(^{2+}\) signals than the Ib terminals. During a train of APs, the postsynaptic Ca\(^{2+}\) signal increased but remained localized to the postsynaptic membrane. In addition, we showed that the plasma membrane Ca\(^{2+}\)-ATPase (PMCA) played a role in extruding Ca\(^{2+}\) from the postsynaptic region of the muscle. *Drosophila melanogaster* has a single PMCA gene, predicted to give rise to various isoforms by alternative splicing. Using RT-PCR, we detected the expression of multiple transcripts in muscle and nervous tissues; the physiological significance of the same is yet to be determined.

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IT IS CLEAR that postsynaptic Ca\(^{2+}\) signals play an important role in regulating synaptic function. The strongest evidence for this comes from studies of long-term potentiation (LTP) and long-term depression (LTD), which are triggered by increases in postsynaptic intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) (Malenka et al. 1988; Neveu and Zucker 1996; Yang et al. 1999). Postsynaptic Ca\(^{2+}\) signals may also play an important role in synaptic homeostasis in cultured mammalian neurons (Thiagarajan et al. 2005; Wierenga et al. 2005) and at the *Drosophila* neuromuscular junction (NMJ). At the *Drosophila* larval NMJ, transgenic larvae with reduced sensitivity to glutamate receptor (GluR) mutants (Haghighi et al. 2003). Postsynaptic Ca\(^{2+}\) signals have also been proposed to influence receptor sensitivity and protein synthesis. At the *Drosophila* NMJ postsynaptic Ca\(^{2+}\) has been shown to regulate the sensitivity and synaptic localization of the postsynaptic receptor GluRIIA (Davis et al. 1998; Morimoto et al. 2010). For both the mammalian central nervous system (CNS) and the *Drosophila* NMJ, protein synthesis occurred locally near the postsynaptic membrane and was regulated by synaptic activity (Ouyang et al. 1999; Sigrist et al. 2000; Steward et al. 1998; Tang and Schuman 2002). In the mammalian CNS this postsynaptic protein synthesis appeared to be regulated by postsynaptic Ca\(^{2+}\) (Ouyang et al. 1997; Raymond et al. 2000; Scheetz et al. 2000; Sutton and Schuman 2005), and this could be the case for the *Drosophila* NMJ as well. Also, *Drosophila* larval muscles are known to contain two Ca\(^{2+}\)-activated K\(^+\) channels (Singh and Wu 1989), and these could also be regulated by postsynaptic Ca\(^{2+}\).

In *Drosophila* larvae, Ca\(^{2+}\) entry at the postsynaptic membrane has been detected by fusing Ca\(^{2+}\) reporters to the PDZ-interaction domain of the Shaker K\(^+\) channel (Guerrero et al. 2005; Peled and Isacoff 2011); these techniques were sensitive to increases in Ca\(^{2+}\) at the postsynaptic membrane. We injected Ca\(^{2+}\) indicators into the muscle to examine the full extent of the Ca\(^{2+}\) signal in the cytoplasm during synaptic transmission. Using this technique, we were able to observe quantal Ca\(^{2+}\) transients produced by spontaneous transmitter release and the transients produced by evoked transmitter release. To determine whether the Ca\(^{2+}\) signals were correlated with synaptic efficacy, we compared the postsynaptic Ca\(^{2+}\) transients produced by the Is and Ib terminals. Generally, Is terminals produce larger EPSPs than Ib terminals (Kurdyak et al. 1994; Lnenicka and Keshishian 2000), and also the quantal size is greater for Is terminals (Karunanithi et al. 2002).

We investigated whether Ca\(^{2+}\) extrusion by the plasma membrane Ca\(^{2+}\)-ATPase (PMCA) played a role in shaping these postsynaptic Ca\(^{2+}\) signals. Previously we showed that PMCA was localized to the *Drosophila* larval NMJ (Lnenicka et al. 2006). We demonstrated that PMCA is present at the muscle membrane and is involved in postsynaptic Ca\(^{2+}\) extrusion. Alternative splicing of the PMCA messenger RNA was known to produce various isoforms in mammals (Strehler and Zacharias 2001), and the same was predicted for the single gene in *Drosophila melanogaster* (Rhead et al. 2010). Of the six predicted splice variants in *Drosophila*, it was not known which isoforms were actually expressed and whether there were any tissue-specific expression patterns. We addressed this with a reverse transcriptase-polymerase chain reaction (RT-PCR) approach.

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**MATERIALS AND METHODS**

**Loading Ca\(^{2+}\) indicators.** Changes in \([\text{Ca}^{2+}]\), were measured in muscle fibres in wandering third-instar larvae with either Oregon Green BAPTA-1 (OGB-1) or rhod-2. We examined muscle fibres 4 of segment 3 or 4 in either Canton S (CS) larvae or the progeny of a cross between P[elav-GAL4/C155 and UAS-GFP/S65T]. For CS larvae, the muscle fibres were impaled with intracellular electrodes containing 20 mM OGB-1, hexapotosmum salt (Molecular Probes, Eugene, OR); for larvae with green fluorescent protein (GFP)-labeled motor terminals, muscles were impaled with intracellular electrodes containing 20 mM OGB-1, hexapotosmum salt (Molecular Probes) into motor terminals was as previously described (Lnenicka and Keshishian 2000). Briefly, the synaptic boutons of the segment 3 or 4 larvae with green fluorescent protein (GFP)-labeled motor terminals, motor terminals were visualized with DIC and penetrated by passing hyperpolarizing current, typically 20 to 30 nA for ~15–20 min. All \([\text{Ca}^{2+}]\) measurements were performed in HL3 saline containing 1 mM Ca\(^{2+}\).

**Measurement of \([\text{Ca}^{2+}]i\), changes.** Terminals and muscle fibres were imaged with an upright, fixed-stage BH2 microscope (Olympus) equipped with epifluorescence, differential interference contrast (DIC), a water-immersion \(\times 40\) Zeiss lens (NA 0.75), and a digital cooled charge-coupled device (CCD) camera (CoolSNAP HQ, Photometrics, Tucson, AZ). Excitation illumination from a 75-W xenon arc lamp was passed through a Lambda-10 Optical Filter Changer (Sutter Instrument, Novato, CA). To image GFP-labeled terminals or OGB-1 fluorescence, we used a 480 \pm 15-nm bandpass excitation filter, a 500-nm dichroic mirror, and a high-pass 515-nm emission filter. Images were imaged with an upright, fixed-stage BH2 microscope (Olympus, Japan). Measurement of \([\text{Ca}^{2+}]i\) changes were compared with paired \(t\)-tests of the area covered by single Ca\(^{2+}\) transients, the areas were compared with paired \(t\)-tests for all experiments, the postsynaptic Ca\(^{2+}\) transients were measured by calculating the percent change in fluorescence (\(\Delta F/\text{F}\); 100 \times (fluorescence - resting fluorescence)/resting fluorescence). Metaphor 6.1 software (Universal Imaging, Downingtown, PA) was used for image acquisition and to map a two-dimensional matrix of \(\Delta F/\text{F}\). Sigmaplot 10.0 (SPSS, Plover, WI) was used to produce three-dimensional graphs of the \(\Delta F/\text{F}\) values. Single exponentials were fit to the \(\Delta F/\text{F}\) decay (r \(\geq 0.98\)) to determine the \([\text{Ca}^{2+}]i\), decay time constant \((\tau)\). To obtain the total \(\Delta F/\text{F}\) (% \(\mu\text{M}\)) for the Ca\(^{2+}\) transients, the area covered by the \(\Delta F/\text{F}\) was multiplied by the average \(\Delta F/\text{F}\). To compare the area covered by single Ca\(^{2+}\) transients, the areas were converted to equivalent diameters: diameter \(= (2\text{area}/\pi)^{1/2}\). Means were compared with paired \(t\)-tests.

For the frequency histograms of equivalent diameters, univariate kernel density smoothing functions were implemented in order to enhance the visualization of the equivalent diameter variable. Plotting of such nonparametric kernel density functions is recommended as a way of estimating shapes of complex univariate distributions (Mannoldn and Braun 2007). The Gaussian kernel was employed with Silverman bandwidth (Silverman 1986) as implemented in R (version 2.11.1). Frequency histograms were plotted with R, which permits overlaying the kernel density function while also showing all raw data points as a Rugplot; this provided a thoroughly informative graph.

**Motor terminal staining and immunocytochemistry.** The method for injecting Lucifer yellow or Lucifer yellow cadaverine biontin-X (Molecular Probes) into motor terminals was as previously described (Lnenicka and Keshishian 2000). Briefly, the synaptic boutons of the prefixed larval preparation were visualized with DIC and penetrated with microelectrodes filled with 5–10% Lucifer yellow. The terminals were filled with Lucifer yellow by briefly passing hyperpolarizing current; then fixation was continued in 4% formaldehyde, and the PMCA was immunostained. Procedures for immunocytochemistry were similar to those used in an earlier study (He et al., 2009). Briefly, the larval preparations were incubated overnight (at 4°C) with anti-PMCA (Lnenicka et al. 2006) and incubated for 5 h (at room temperature) with Alexa Fluor 546 donkey anti-rabbit IgG (Invitrogen, Carlsbad, CA) (1:200 dilution). The larvae were mounted in SlowFade Gold (Molecular Probes) and viewed under the Zeiss LSM 510 confocal microscope. Fluorescence was visualized with a 10× oil lens with a BP 505–530 filter and a 543 laser with a LP560 filter. Stacks of images were acquired from segments 3 and 4 of muscle fiber 6.

**Western blots and RT-PCR.** For Western blots, larval brain and muscle proteins were separated by SDS-PAGE and electroblotted to nitrocellulose membranes (Laemmli 1970; Towbin et al., 1979). Briefly, brains were isolated from third-instar larvae and homogenized in loading buffer containing reducing agent (NuPAGE kit, Invitrogen). For muscle tissue samples, the brains were first removed from dissected larvae, and then the muscle fibers were scraped off from the underlying body wall. The rest of the procedure was as previously described (Lnenicka et al. 2006).

The annotated *Drosophila* PMCA gene CG42314 (http://genome.ucsc.edu/) is predicted to yield six different mRNA transcripts: CG42314-RC, -RD, -RE, -RF, -RG, and -RH. In terms of the coding sequence there is no difference between transcripts CG42314-RE and -RG, and hence their translation products are identical.

Starting from the 5’ end, the first 14 exons are identical among all six transcripts. They differ only in the last few exons (1 to 3) at their respective 3’ ends, which encode the COOH termini of their translated proteins. To determine which of these transcripts were present we devised an RT-PCR approach. The forward primer (primer A: 5’-CAGATGGATGAACTCGGAGG-3’) is from exon 14 and hence was common to all transcripts. The reverse primers were designed to yield PCR products of varying sizes depending on which transcripts were expressed. Primer 1 (5’-CTCGACTTTCGATGTTAAGG-3’) is from exon 15, which is shared by transcripts CG42314-RE, -RD, -RF, and -RH, but because of differences in the total number of exons among these transcripts, PCR products of varying sizes are obtained: 396 bp (CG42314-RE and -RG: 16 exons in total), 1 kb (CG42314-RH: 17 exons in total), and 300 bp (CG42314-RC: 15 exons in total). Primers 2 (5’-CGGTAATAACCAACATTAACAGAG-3’) is from exon 16b, exclusive to transcript CG42314-RF, and yielded a 1,037-bp PCR product. Primer 3 (5’-GGCGACCTTGGCGAGTATAATTGC-3’) was from exon 15, exclusive to transcript CG42314-RD, and yielded a 225-bp PCR product.

Total RNA was isolated from brains, muscle fibers, and whole larvae of Canton S third-instar females and males. Brain and muscle fiber samples were obtained as mentioned above. The tissues were first homogenized with a Brinkmann Polytron PT 3000 homogenizer (Kinematica, Littau/Lucerne, Switzerland), and then total RNA was isolated with the RNeasy Mini Kit (Qiagen, Valencia, CA). RT-PCR was performed with SuperScript III One-Step RT-PCR with Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA). For each RT-PCR, 50 ng of total RNA was used as template, and after incubation at 55°C for 35 min (for reverse transcription), the PCR was run in the presence of forward primer A and either of the reverse primers (primer 1, 2, or 3). An initial denaturation step at 94°C for 2 min was followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 60°C for 30 s, and extension at 68°C for 1.5 min. The DNA products obtained were subjected to electrophoresis using 1% Tris-borate-EDTA (TBE) agarose gels, and the 2-log DNA ladder (New England Biolabs, Ipswich, MA) was used as the size standard.

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RESULTS

Discrete postsynaptic Ca\textsuperscript{2+} transients seen along the terminal during evoked transmitter release. To examine postsynaptic Ca\textsuperscript{2+} transients, we injected muscle fibers with the Ca\textsuperscript{2+} indicator rhod-2. We used muscle fiber 4 because of its large size and the fact that the two type I motor terminals, Ib and Is, are usually well separated; this allowed us to identify the Ca\textsuperscript{2+} signals produced by each terminal. To clearly view the terminals, we used the bipartite GAL4-UAS system (Brand and Perrimon 1993) to express GFP in neurons; an elav-GAL4 line was crossed to a UAS-GFP line. Both the rhod-2 fluorescence from muscle and the GFP fluorescence from terminals were viewed in the same experiment (Fig. 1A).

When single APs were evoked in the nerve, we observed discrete Ca\textsuperscript{2+} signals in the postsynaptic region of the muscle along the synaptic terminal. To determine the location of these postsynaptic Ca\textsuperscript{2+} transients in relation to the terminal, we mapped the Ca\textsuperscript{2+} transient peaks on the terminal (Fig. 1B). We assume that the peak increase in [Ca\textsuperscript{2+}]\textsubscript{i} would be roughly centered on the postsynaptic receptor field that was activated by the neurotransmitter. Thus the peaks should identify the sites of transmitter release. As expected, we found considerable variability in the sites of transmitter release during successive APs; however, most peaks occurred at synaptic boutons (Fig. 1B). On average, we found that boutons had an associated Ca\textsuperscript{2+} transient for 80% of the stimulations and bottlenecks only showed Ca\textsuperscript{2+} transients 16% of the time (Fig. 1C). Thus transmitter release usually occurs at boutons but can also occur at bottlenecks. Also, we found that the branch point showed significant transmitter release; Ca\textsuperscript{2+} transients were found at the branch point for 96% of the stimulations. This was the only branch point examined, so we do not know whether this applies to all branch points.

Discrete Ca\textsuperscript{2+} transients seen during evoked transmitter release often result from a single quantum of transmitter. We conducted a quantal analysis for the Ca\textsuperscript{2+} transients seen during evoked transmitter release and characterized the Ca\textsuperscript{2+} transients produced by spontaneous transmitter release. We were able to observe spontaneous Ca\textsuperscript{2+} transients; however, it was difficult to obtain a large number of them since we could only record for short periods and we were limited to a small region of the terminal. However, we found it easier to record spontaneous events from Is terminals than Ib terminals. Thus we used the Is terminals on muscle fiber 4 for this study. In these experiments, we used OGB-1 as our Ca\textsuperscript{2+} indicator because of its brighter fluorescence, and thus we could not use GFP-labeled terminals; instead, we viewed the terminals with DIC.

We analyzed three experiments and found very consistent results. First, we compared the amplitude of the evoked Ca\textsuperscript{2+} transients to the spontaneous Ca\textsuperscript{2+} transients. For our analyses, we only considered “single Ca\textsuperscript{2+} transients” defined as having

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Fig. 1. Postsynaptic Ca\textsuperscript{2+} transients seen during single presynaptic action potentials (APs). A, top: 3-dimensional (3D) graph of the Ca\textsuperscript{2+} transients seen in muscle fiber 4 resulting from a single AP in the Ib terminal. This image was taken at the time that the postsynaptic response was at its peak. The x- and y-axes represent distance, and the z-axis is the change in rhod-2 fluorescence; pseudocolor calibration shows % change in fluorescence (ΔF/F). Bottom: green fluorescent protein (GFP)-labeled Ib terminal on the same muscle fiber. The terminal is divided into 5 boutons and bottlenecks as well as a branch point. Calibration bar represents 10 μm. The distance scale for this image is the same as the above 3D graph; however, the graph is tilted to allow the Ca\textsuperscript{2+} transients to be seen more clearly. B: locations of the Ca\textsuperscript{2+} transient peaks for 5 consecutive APs are represented by black dots and presumably represent the sites of transmitter release. C: based on the muscle Ca\textsuperscript{2+} transients, we calculated the % of trials that transmitter was released at the various regions of the terminal for 110 presynaptic APs. The boutons showed 5-fold more transmitter release than the bottlenecks, and there was also considerable transmitter release at the branch point.
a single peak and no overlap with other Ca\textsuperscript{2+} signals. The peak amplitudes of the spontaneous and evoked single Ca\textsuperscript{2+} transients were very similar in all three experiments (Fig. 2, A and B). Thus it first appeared that the single Ca\textsuperscript{2+} transients seen during evoked transmitter release represented the release of a single quantum of transmitter. However, the total ΔF/F (Ca\textsuperscript{2+} transients volume) of the evoked single Ca\textsuperscript{2+} transients was on average 55% larger than that for spontaneous Ca\textsuperscript{2+} transients (Fig. 2C) because the former often covered a larger area. Thus some of the evoked single Ca\textsuperscript{2+} transients could represent two nearby quantal Ca\textsuperscript{2+} transients.

To test this, we examined frequency histograms of the diameters for the evoked single Ca\textsuperscript{2+} transients and compared them with the mean diameter of the spontaneous Ca\textsuperscript{2+} transients (Fig. 2D). We assumed that two quanta of transmitter released side by side would activate twice the field of postsynaptic receptors as a single quantum and produce a larger-diameter Ca\textsuperscript{2+} transient. This appeared to be the case; the histogram of diameters appeared bimodal, with the larger mode approximately twice that of the smaller mode. In addition, the diameter of a spontaneous Ca\textsuperscript{2+} transient was similar to the diameter for the smaller mode. This supports our proposal that the evoked single Ca\textsuperscript{2+} transients likely represented one or two quanta of transmitter. It should be noted that the amplitude of these evoked Ca\textsuperscript{2+} transients did not show a bimodal distribution, indicating that the quantal Ca\textsuperscript{2+} transients did not completely overlap (Fig. 2E). We compared the decay rates of the spontaneous and evoked single Ca\textsuperscript{2+} transients and found they were similar: decay time constants for Ca\textsuperscript{2+} transients selected from experiment 2 were 53 ± 1 ms (n = 12) and 58 ± 4 ms (n = 65) for the spontaneous and evoked Ca\textsuperscript{2+} transients, respectively.

The amplitude and diameter of the Ca\textsuperscript{2+} transients varied among experiments and likely resulted from variability in OGB-1 concentration ([OGB-1]). Higher concentrations of a mobile Ca\textsuperscript{2+} indicator would be expected to decrease the amplitude and increase the diameter of the Ca\textsuperscript{2+} transients (Neher 1995; Wagner and Keizer 1994). Resting fluorescence presumably reflected the [OGB-1], and it was 1,071, 737, and 629 arbitrary units (AU) for experiments 1, 2, and 3, respectively, indicating that [OGB-1] was greatest in experiment 1 and lowest in experiment 3. As predicted, the amplitudes of the spontaneous Ca\textsuperscript{2+} transients (6.8%, 11.3%, and 14.4%) were negatively related to [OGB-1] and their diameters (8.2 μm, 4.6 μm, and 3.7 μm) were positively related to [OGB-1]. In the absence of indicator, we assume that the quantal Ca\textsuperscript{2+} signal diameter was <3.7 μm since this value was seen for experiment 3 with the lowest [OGB-1].

Terminals with differences in evoked postsynaptic response and quantal size show differences in postsynaptic Ca\textsuperscript{2+} transients. We compared the postsynaptic Ca\textsuperscript{2+} transients produced by the Is and Ib terminals. Since the EPSP amplitudes and quantal size are greater for Is than Ib terminals (Atwood et al. 1993; Karunanithi et al. 2002; Kurdyak et al. 1994; Lnenicka and Keshishian 2000), one would expect greater Ca\textsuperscript{2+} influx at the postsynaptic membrane of the Is terminals compared with the Ib terminals. We measured Ca\textsuperscript{2+} transients at both Is and Ib terminals in the same muscle fiber by using rhod-2-loaded muscle fiber 4 and GFP-labeled terminals (Fig. 3A).

We first determined whether the size of the Ca\textsuperscript{2+} signal per terminal length was greater for Is compared with Ib terminals. For this purpose, we measured the total ΔF/F for the terminal and divided this value by terminal length. For all four experiments, this value was three- to fourfold greater for Is than Ib terminals (Fig. 3B). These differences in the Ib and Is Ca\textsuperscript{2+} transients could not be accounted for by the number of boutons per terminal since they were very similar in all four experiments (Ib boutons, Is boutons): experiments 1 (3, 4), 2 (5, 4), 3 (7, 7), and 4 (7, 6).

Next we determined whether there were differences in the size of quantal Ca\textsuperscript{2+} transients. Since we previously showed that there was a strong correlation between the peak amplitudes of the spontaneous and evoked single Ca\textsuperscript{2+} transients for Is terminals, we measured the amplitude of the evoked single Ca\textsuperscript{2+} transients (Fig. 3A) at the Is and Ib terminals. On average the peak amplitude of the evoked single Ca\textsuperscript{2+} transients produced at the Is terminals was 125% larger than at the Ib terminals for the same muscle fiber (Fig. 3C). This suggests that quantal Ca\textsuperscript{2+} transients produced at the Is terminals are larger than those produced at the Ib terminals. Finally, for experiment 1, we examined whether the Ca\textsuperscript{2+} transients produced at the two terminals differed in their decay rates. The decay time constants were very similar for the Ib (170 ± 10 ms, n = 61) and Is (190 ± 15 ms, n = 58) terminals.

Effect of repetitive stimulation on postsynaptic Ca\textsuperscript{2+} signal. We were interested in determining whether repetitive nerve stimulation would result in a buildup of postsynaptic Ca\textsuperscript{2+}. For three experiments we delivered a train of APs at 10 Hz for 5 s and compared the Ca\textsuperscript{2+} transients produced by the first stimulation (Fig. 4A) with those seen at the plateau (Fig. 4B). Relative to the first AP, repetitive stimulation resulted in an approximate fivefold increase in the total ΔF/F of the postsynaptic Ca\textsuperscript{2+} transients produced at the Is terminals (Fig. 4C). This increase in [Ca\textsuperscript{2+}], along the terminal was more uniform than that seen for the first stimulation; however, the postsynaptic Ca\textsuperscript{2+} transients remained localized to the NMJ (Fig. 4B).

Plasma membrane Ca\textsuperscript{2+} ATPase is important for clearance of postsynaptic Ca\textsuperscript{2+}. Ca\textsuperscript{2+} clearance mechanisms likely play an important role in shaping the postsynaptic Ca\textsuperscript{2+} signals. We determined whether PMCA played a role in Ca\textsuperscript{2+} clearance. Our previous work showed localization of a PMCA antibody to the larval NMJ (Lnenicka et al. 2006). Since physiological studies showed that PMCA was responsible for Ca\textsuperscript{2+} extrusion from the presynaptic terminals (Lnenicka et al. 2006), we assumed that PMCA was only localized to the presynaptic membrane. Upon closer examination, there was evidence that PMCA was also found at the postsynaptic membrane. We examined the NMJ after injecting the motor terminals with Lucifer yellow and applying the PMCA antibody. The double staining showed that much of the PMCA staining formed an outer border to that of Lucifer yellow (Fig. 5). This staining occurs at the site of the subsynaptic reticulum (SSR), an infolding of the muscle membrane reaching ~0.5–0.7 μm into the muscle (Jia et al. 1993). Staining of proteins found in this postsynaptic membrane would result in a border surrounding the terminal as seen here. A similar staining pattern was seen for Discs-large (DLG), a protein found in the postsynaptic membrane of these NMJs (Guan et al. 1996; Lahey et al. 1994).
Fig. 2. Comparison of the evoked and spontaneous postsynaptic Ca\(^{2+}\) transients at Is terminals of muscle fiber 4. 

A: postsynaptic Ca\(^{2+}\) signals recorded along the Is terminal from muscle fibers loaded with Oregon Green BAPTA-1 (OGB-1). Left: representative 3D graphs of Ca\(^{2+}\) transients seen in the muscle during single APs in the Is terminal. Right: representative 3D graphs of spontaneous Ca\(^{2+}\) transients seen in the muscle in the absence of nerve stimulation. All images represent the postsynaptic responses at their peaks. The x- and y-axes represent distance, and the z-axis is the change in OGB-1 fluorescence. For all 3D graphs, pseudocolor calibration shows the \(\Delta F/F\) (%) and the calibration bar represents 20 µm. 

B: the peak amplitude of the spontaneous Ca\(^{2+}\) transients was very similar to the evoked single Ca\(^{2+}\) transients. The means for spontaneous and evoked Ca\(^{2+}\) transient were not significantly different (\(P = 0.92\)); \(n\) values shown in parentheses represent the number of Ca\(^{2+}\) transients. 

C: the total \(\Delta F/F\) of the evoked single Ca\(^{2+}\) transients was on average 55% larger than that of the spontaneous Ca\(^{2+}\) transients; \(n\) values are the same as in B. The means for spontaneous and evoked Ca\(^{2+}\) transient were significantly different (\(P = 0.02\)). 

D and E: frequency histograms of the equivalent diameter (µm) and peak amplitude (%) respectively, for the evoked single Ca\(^{2+}\) transients from the above 3 experiments. In D, kernel densities used Gaussian Kernel with Silverman bandwidth as implemented in R. Individual data points are shown as the Rugplot. For each experiment, the spontaneous Ca\(^{2+}\) transient mean equivalent diameter (µm) is shown in parentheses and the arrow marks its position with respect to the evoked Ca\(^{2+}\) transients. For B–E, experiments 1, 2, and 3 are shown left to right.
yield identical translation products, and hence there are only three proteins: the larger band RF, the smaller band RD, and the band between RF and RD, which yields five possible distinct PMCA proteins. To identify which specific isoforms were present in the larvae, and to see whether there were any sex- or tissue-specific expression patterns, we further characterized the expression of PMCA by RT-PCR using primers that yielded distinct products for each of the PMCA mRNA splice forms (Fig. 7B); for details refer to MATERIALS AND METHODS. Regardless of the source of total RNA, identical results were obtained. The primers designed to detect the RE/RG, RF, and RD transcripts yielded bands of the expected sizes (396 bp, 1,037 bp, 225 bp, respectively; Fig. 7C), whereas those designed to detect transcripts RC and RH yielded no product, indicating that of the six only these four transcripts are expressed in larvae with no difference between sexes, muscle, or nervous tissues. The predicted molecular masses of the proteins derived from these transcripts are 132.9 kDa each from CG42314-RE and -RG, 138.7 kDa from CG42314-RF, and 123.4 kDa from CG42314-RD. Thus the larger band present on our Western blot likely represented the translated products of RE, RF, and RG and the smaller band RD.

**DISCUSSION**

**Ca**\(^{2+}\) transients were produced by evoked and spontaneous transmitter release. We examined the postsynaptic Ca\(^{2+}\) signals at the larval NMJ and observed Ca\(^{2+}\) transients produced by evoked and spontaneous transmitter release. At the *Drosophila* NMJ, the postsynaptic GluRs are known to be Ca\(^{2+}\) permeable (Chang et al. 1994). These Ca\(^{2+}\) transients were likely produced by Ca\(^{2+}\) influx through the GluRs and not the voltage-dependent Ca\(^{2+}\) channels since they are seen during spontaneous transmitter release, which produces small postsynaptic depolarization, and they are localized, even during synaptic depolarization, and they are localized, even during

![Fig. 3. Comparison of the evoked postsynaptic Ca\(^{2+}\) transients seen along the Is and Ib motor terminals on muscle fiber 4. A, top: 3D graph showing representative evoked Ca\(^{2+}\) transients seen in a rhod-2-loaded muscle fiber during a single AP in both Is and Ib terminals. The x- and y-axes represent distance, and the z-axis is the change in rhod-2 fluorescence. Pseudocolor calibration shows the ∆F/F (%). The graph is tilted to allow the Ca\(^{2+}\) transients to be seen more clearly. Single Ca\(^{2+}\) transients are marked by arrows. Bottom: GFP-labeled nerve terminals for the same muscle fiber. The larger Ib boutons (left) and the smaller Is boutons (right) are indicated by arrows. Calibration bar represents 10 µm. B: for each experiment ~50 images of the evoked postsynaptic Ca\(^{2+}\) signals were averaged together to obtain a single image, from which the total ∆F/F for the evoked Ca\(^{2+}\) transients at the Is and Ib terminals was calculated and then divided by terminal length. The total ∆F/F per micrometer of terminal length was 3- to 4-fold greater at Is compared with Ib terminals. The means for Ib and Is were significantly different (P = 0.007). C: the peak amplitudes of the single Ca\(^{2+}\) transients were ~125% greater at Is than Ib terminals; n values are shown in parentheses. The means for Ib and Is were significantly different (P = 0.01). For B and C, experiments 1, 2, 3, and 4 are shown left to right.

their decay before and after inhibiting PMCA. PMCA exchanges extracellular proton(s) for intracellular Ca\(^{2+}\) (Niggli et al. 1982; Smallwood et al. 1983; Xu and Roufogalis 1988), and it can be inhibited by raising the external pH (Kratje et al. 1982; Smallwood et al. 1983; Xu and Roufogalis 1988), and their decay before and after inhibiting PMCA. PMCA exchanges extracellular proton(s) for intracellular Ca\(^{2+}\) (Niggli et al. 1982; Smallwood et al. 1983; Xu and Roufogalis 1988), and it can be inhibited by raising the external pH (Kratje et al. 1982; Smallwood et al. 1983; Xu and Roufogalis 1988), and

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trains of APs. In addition, an earlier study showed an increase in postsynaptic Ca²⁺/H⁺ during synaptic transmission even when Ca²⁺/H⁺ release from internal stores was inhibited and the postsynaptic membrane was voltage clamped (Guerrero et al. 2005). The Ca²⁺ must spread considerably after entering at a synapse, since we found that quantal Ca²⁺ signals have a diameter of ~3.7 μm and these synapses have a diameter of ~0.6 μm (Atwood et al. 1993). Similar Ca²⁺ signals may be generated at other NMJs; e.g., at the vertebrate and crustacean NMJs the postsynaptic acetylcholine and glutamate receptors allow Ca²⁺ entry (Adams et al. 1980; Decker and Dani 1990; Dekin 1983; Dudel 1974; Onodera and Takeuchi 1976; Vernino et al. 1994).

We observed the occurrence of postsynaptic quantal Ca²⁺ signals at the larval NMJ under physiological conditions. A similar finding was recently reported: Ca²⁺ influx through the postsynaptic GluRs at the larval NMJ was demonstrated during spontaneous transmitter release (Peled and Isacoff 2011). In the mammalian CNS, quantal Ca²⁺ transients produced by NMDA receptors (NMDARs) have been observed in the absence of external Mg²⁺ (Murphy et al. 1994); these NMDAR-mediated spontaneous Ca²⁺ transients might also occur under physiological conditions (Sutton and Schuman 2005). Spontaneous transmitter release plays an important role in synaptic regulation and could involve quantal Ca²⁺ transients. In the rat hippocampus, spontaneous vesicular glutamate release is im-

![Fig. 4. Increase in postsynaptic intracellular Ca²⁺ concentration ([Ca²⁺]i) seen during repetitive nerve stimulation (10-Hz train for 5 s). Representative 3D graphs of postsynaptic Ca²⁺ transients seen during the 1st stimulation (A) and at the plateau (B), i.e., ~4 s from the start of the train, are shown. The x- and y-axes represent distance, and the z-axis is the change in OGB-1 fluorescence. Calibration bar for length represents 10 μm, and the pseudocolor calibration representing ΔF/F (%) is the same for both graphs. The graphs are tilted to allow the Ca²⁺ transients to be seen more clearly. C: representative time course of the postsynaptic Ca²⁺ transient produced during the train. The values for the total ΔF/F of the muscle Ca²⁺ transient were plotted as a function of time. Note that the train begins at ~0.5 s. The total ΔF/F for the 1st stimulation is marked by an arrow.](http://jn.physiology.org/)

![Fig. 5. Plasma membrane Ca²⁺-ATPase (PMCA) is present at the postsynaptic membrane.](http://jn.physiology.org/)

A: image of a nerve terminal injected with Lucifer yellow. B: staining with rabbit anti-PMCA and Alexa Fluor 546 donkey anti-rabbit IgG as primary and secondary antibodies. C: superimposition of the 2 images revealed that the PMCA stained largely outside of the presynaptic terminal, presumably staining the muscle subsynaptic reticulum. Calibration bar represents 5 μm and applies to all 3 images.)
portant for the maintenance of dendritic spines on CA1 pyramidal cells (McKinney et al. 1999). In cultured hippocampal neurons, miniature synaptic events are known to regulate local dendritic protein synthesis and GluR insertion at the postsynaptic membrane (Sutton et al. 2004, 2006). At the Drosophila NMJ, spontaneous transmitter release was shown to induce GluR clustering during development (Saitoe et al. 2001). Also, a reduction in the sensitivity of postsynaptic receptors to spontaneous transmitter release triggers a rapid, compensatory increase in transmitter release (Frank et al. 2006).

During evoked transmitter release, many of the Ca\(^{2+}\) signals represented the release of one quanta of transmitter and could be used to map transmitter release along the terminal. In this initial study, we observed that most release was seen at synaptic boutons and occurred occasionally at bottlenecks. This is consistent with the serial electron microscopic reconstructions of the terminals that show most synapses at boutons, but a few at bottlenecks (Atwood et al. 1993). An optical quantal analysis has previously been performed at the dendritic spines of rat CA1 pyramidal neurons by imaging the postsynaptic Ca\(^{2+}\) transients (Oertner et al. 2002). A similar optical quantal analysis may prove useful in future studies of the NMJ.

For the muscle fiber 6 Ib terminal, distal boutons released more transmitter than proximal boutons, which was attributed to greater presynaptic Ca\(^{2+}\) influx for distal boutons (Guerrero et al. 2005). In Fig. 1, there was no apparent gradient of transmitter release for the muscle fiber 4 Ib terminal. Also, in the other experiments presented here we did not see a clear gradient of transmitter release for the muscle 4 Is or Ib terminals (data not reported). Thus the muscle fiber 4 terminals may differ from those seen on muscle fiber 6. These results are consistent with our measurements of presynaptic Ca\(^{2+}\) transients: both Is and Ib terminals on muscle fiber 6 showed a clear gradient of Ca\(^{2+}\) influx, but a gradient was not apparent for the Is and Ib terminals on muscle fiber 4 (He et al. 2009; Lnenicka et al. 2006).

Our data suggest that the release of two quanta of transmitter from a single synapse was a rare event. Presumably, if two vesicles were released from the same synapse, we should see a doubling of the Ca\(^{2+}\) transient amplitude; however, the sample of Ca\(^{2+}\) transient amplitudes in Fig. 2 did not show any amplitudes that were clearly twice the mode. This might simply result from the low probability of release for a single synapse (Atwood et al. 1993). These results would also be seen if the postsynaptic GluRs were saturated by a single vesicle of transmitter. This does not seem to be the case since a previous study reported an increase in quantal size upon increasing the vesicular glutamate content (Daniels et al. 2004). It does appear that nearby synapses at the same bouton show simultaneous transmitter release. This can be seen from the closely spaced peaks (Fig. 1A) and the observation that some of the evoked Ca\(^{2+}\) transients with single peaks appear to represent the overlap of two quantal Ca\(^{2+}\) transients.

Stronger synapses produce greater Ca\(^{2+}\) signals. The Is terminals are known to produce larger EPSPs than the Ib terminals, which is consistent with the idea that stronger synapses produce greater Ca\(^{2+}\) signals.

Fig. 6. Effect of PMCA inhibition by high pH on Ca\(^{2+}\) clearance from the muscle. A: representative 3D graphs of the Ca\(^{2+}\) transient showing its decay after a 10-Hz train for normal pH 7.3 (bottom) and high pH 8.8 (top) at end of the train (I), 150 ms after the end (II), and 300 ms after the end (III). The x- and y-axes represent distance, and the z-axis is the change in OGB-1 fluorescence; the calibration bar for length (20 \(\mu\)m) and the pseudocolor calibration for \(\Delta F/F\) are the same for all graphs. B: decay of the Ca\(^{2+}\) transients at the postsynaptic membrane for normal PMCA function (●) and PMCA inhibition (○). Arrows and Roman numbers show the time points at which the 3D graphs in A were obtained. C: the decay time constant (\(\tau\)) for the Ca\(^{2+}\) transient produced by the 10-Hz train was ~3-fold greater in high pH than normal pH. The means were significantly different (\(P = 0.01\); n value equals 3 for all.)
terminals (Kurdyak et al. 1994; Lnenicka and Keshishian 2000). If postsynaptic Ca\(^{2+}\)/H\(^{11001}\) is being used to gauge synaptic efficacy and trigger mechanisms for synaptic homeostasis, then one would expect the Is terminals to produce greater postsynaptic Ca\(^{2+}\)/H\(^{11001}\) signals. We report that the Ca\(^{2+}\)/H\(^{11001}\) signal per terminal length is in fact three- to fourfold greater for Is relative to Ib terminals. At the Drosophila NMJ, the mean vesicle volume is 68% greater for Is synaptic vesicles than Ib, and the miniature EPSP amplitude is 53% greater at Is than Ib boutons, indicating that a greater amount of glutamate is released from Is synaptic vesicles (Karunanithi et al. 2002). Thus the Is synaptic vesicles would be expected to activate a larger number of postsynaptic receptors and produce a larger postsynaptic Ca\(^{2+}\) signal. A comparison of the evoked single Ca\(^{2+}\) transients showed that the Is quantal Ca\(^{2+}\) signal appears to be twice that of the Ib signal. If differences in quantal Ca\(^{2+}\) transients accounted for a twofold difference in evoked Ca\(^{2+}\) transients, then transmitter release was 1.5- to 2-fold greater per length of Is terminal compared with Ib terminals.

**PMCA plays a role in extruding postsynaptic Ca\(^{2+}\).** PMCA has been shown to be concentrated at dendritic spines in the mammalian CNS (Burette and Weinberg 2007), where it contributes to Ca\(^{2+}\) extrusion and helps shape the postsynaptic Ca\(^{2+}\) transient (Scheuss et al. 2006; Simons et al. 2009). Our results indicate that PMCA functions to clear Ca\(^{2+}\) from the postsynaptic membrane at the Drosophila NMJ. Other mechanisms may also be involved in Ca\(^{2+}\) clearance from the postsynaptic region of the muscle. For example, the sarcenodoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) has a high affinity for Ca\(^{2+}\) and plays an important role in regulating Ca\(^{2+}\) in skeletal and smooth muscles (Carafoli 1987). There is evidence for Na\(^{+}/Ca\(^{2+}\) exchange activity in invertebrate muscle (Rasgado-Flores et al. 1989; Ruscak et al. 1987), although this has not been examined in Drosophila muscle.
PMCA may be particularly effective at the postsynaptic membrane since the extensive infolding of the SSR provides a large surface area for Ca\(^{2+}\) extrusion. In fact, the function of the SSR is unknown, but it may exist to provide a large surface area for ion extrusion. This could be important for limiting the postsynaptic Ca\(^{2+}\) signals and/or restoring Ca\(^{2+}\) levels in the synaptic cleft. The localization of the PMCA to the postsynaptic membrane could be due to the scaffolding protein Discs-large (DLG). Although DLG is found in the pre- and postsynaptic cell, it is most abundant at the postsynaptic membrane (Lahey et al. 1994). DLG is a homolog of the mammalian PSD-95, and both contain multiple PDZ domains, which can bind other proteins. Mutations in dlg showed that this protein was essential for localizing Fasciclin II and Shaker K channels to the postsynaptic membrane at the larval NMJ (Tejedor et al. 1997; Zito et al. 1997). The COOH terminus of the transcripts CG42314-RD, -RE, and -RG (-T-X-V) (Fig. 8A) match the PDZ binding motif (Kornau et al. 1995), and thus it seems reasonable that they could be localized to the postsynaptic membrane by DLG. This is in agreement with a previous study that proposed that the mammalian PSD-95 recruits PMCA2 to the postsynaptic signaling complex via its PDZ domains (Garside et al. 2009).

PMCA isoforms may show differences in Ca\(^{2+}\) sensitivity and kinetics. In mammals, there are 4 PMCA genes producing over 20 possible splice variants (Strehler and Treiman 2004; Strehler and Zacharias 2001). These proteins are regulated by calmodulin (CaM) binding to their COOH-terminal cytosolic tails (Penniston and Enyedi 1998), which contain the CaM binding domain (James et al. 1988). Alternative splicing at this site results in variants with differential affinity for CaM and hence Ca\(^{2+}\). All identified Drosophila PMCA isoforms differ only at their COOH termini (Fig. 8A); these differences may result from alternative splicing at the CaM binding site and produce differential CaM affinity.

The human CaM binding domain resembles the myosin CaM binding sequence, the IQ motif (Cheney and Mooseker 1992), and this may be the case in Drosophila as well (Fig. 8B). Among the human variants, the IQ consensus is best matched by the human PMCA-2b isoform (Strehler and Zacharias 2001), which is known to be extremely sensitive to CaM and possess elevated basal activity (Elwess et al. 1997; Hilfiker et al. 1994). Of all the Drosophila isoforms, hPMCA-2b’s CaM binding domain is best matched by CG42314-RF, including the two residues (underlined in Fig. 8B) known to be crucial for hPMCA-2b’s greater CaM affinity (Penheiter et al. 2005; Strehler and Zacharias 2001). Thus, relative to the other larval isoforms, CG42314-RF may possess the greatest CaM sensitivity and be activated at lower Ca\(^{2+}\) concentrations.

The other isoforms, CG42314-RE and CG42314-RG, bear no resemblance to the IQ consensus downstream of the splice junction (Fig. 8B). In mammals such isoforms have low CaM affinity but exhibit extremely fast kinetics and are mainly found in excitable cells with rapid Ca\(^{2+}\) increases (Caride et al. 1999; Enyedi et al. 1994). The Drosophila CG42314-RE and CG42314-RG isoforms may also have low CaM affinity but fast kinetics, and DLG may target them to the synapse where

![Fig. 8. Sequence analysis of PMCA isoforms. A: alignment of the COOH-terminal sequence of the 4 Drosophila PMCA isoforms. The identical sequences, CG42314-RE and CG42314-RG, are shown together. The alignment starts with proline (P), residue 1082; prior residues are identical in all the isoforms. The last residue for each of the PMCA isoforms is numbered in parentheses and shown on right. Residues completely conserved in all PMCA isoforms are marked with an asterisk. CG42314-RE is seen to terminate shortly after the splice site and hence is the smallest polypeptide. B: alignment of the IQ domain consensus sequence with the IQ-like sequences of human PMCA-2b and the Drosophila isoforms. The IQ consensus sequence ("x" refers to any amino acid) has been aligned with the various PMCA splice variants starting 4 residues upstream of the splice junction (indicated by a slash). Residues perfectly matching the IQ consensus sequence are shown in bold. CG42314-RF has the best match with the IQ consensus sequence.)](http://jn.physiology.org/doi/abs/10.1152/jn.01060.2010)
they would be particularly effective in clearing the Ca\(^{2+}\) produced during synaptic transmission.

The smallest PMCA isoform, CG42314-RD, may lack Ca\(^{2+}\) regulation and be constitutively active. CG42314-RD terminates near the end of the CaM binding domain and bears no similarity to the IQ consensus downstream of the splice site (Fig. 8). Mammalian isoforms with such features have been identified and are believed to be constitutively active under physiological conditions (Filoteo et al. 2000; Stauffer et al. 1993).

ACKNOWLEDGMENTS

We thank Bruce Dudek for helping us with the Kernel densities and Ruggplots. The elav-GAL4 and UAS-GFP lines were obtained from the Bloomington Drosophila Stock Center.

GRANTS

This work was supported by National Science Foundation Grant IOS-1051605 (G. A. Lnenicka).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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