Fast Calcium Signals in Drosophila Motor Neuron Terminals

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Macleod, G. T., M. Hegström-Wojtowicz, M. P. Charlton, and H. L. Atwood. Fast calcium signals in Drosophila motor neuron terminals. J Neurophysiol 88: 2659–2663, 2002; 10.1152/jn.00515.2002. Drosophila is a powerful model for neuroscientists, but physiological techniques have not kept pace with advances in molecular genetics. We introduce a reliable assay for intracellular calcium dynamics in Drosophila larval motor neuron terminals, and a new physiological solution that improves the longevity of the larval preparation. By loading calcium indicators into motor neuron terminals through cut axons, we obtained a high signal-to-noise ratio with confocal microscopy, and good temporal resolution of calcium-dependent fluorescence changes. We provide an estimate for the resting intracellular calcium concentration, the first description of calcium kinetics for a single action potential (AP), and improved resolution of calcium kinetics during AP trains. The very rapid decay of the calcium signal following a single AP (τ ~ 60 ms) indicates a previously unreported fast calcium extrusion mechanism in Drosophila motor neuron terminals well suited for sustaining physiological processes during the high rates of impulse activity which drive locomotor activity.

INTRODUCTION

As an experimental organism, Drosophila is at the forefront of genetic and molecular research, and in addition affords the opportunity for physiological experimentation. Despite much physiological work on synaptic transmission, progress has been impeded by lack of reliable measurements of the intracellular calcium concentration ([Ca$^{2+}$]). Earlier attempts to measure presynaptic [Ca$^{2+}$], employed membrane permeant Ca$^{2+}$ indicators (Dawson-Scully et al. 2000; Karunanithi et al. 1997; Umbach et al. 1998). However, this technique is capricious, sometimes gives contradictory results, and loads all cell types, generating high background fluorescence. In addition, resolution of presynaptic Ca$^{2+}$ signals for single action potentials (APs) has not been accomplished. To overcome current limitations, we employed a different technique: exposure of cut axons to dextran-conjugated fluorescent Ca$^{2+}$ indicators (O’Donovan et al. 1993). We also designed a new physiological solution in which isolated preparations remain stable for many hours. We have measured resting [Ca$^{2+}$]$_i$ and nerve-evoked Ca$^{2+}$ signals and observed remarkably rapid Ca$^{2+}$ kinetics that enable the neuron terminal to maintain Ca$^{2+}$ homeostasis during high frequency activity. These observations provide the basis for further comparisons with mutant phenotypes.

METHODS

Experiments were performed on Drosophila Canton S wandering third instar larvae, raised on cornmeal agar with dry yeast at 21°C. Larvae were dissected (Jan and Jan 1976) in chilled Schneider’s insect medium (Schneider’s, Sigma, St. Louis MO) and pinned to the Sylgard (Midland, MI) base of a 0.5-ml perfusion bath. Dissections were performed in Schneider’s because it renders the preparation quiescent and stable. After dissection, except where indicated, Schneider’s was replaced with the new physiological solution (Hemolymph-Like No.6 (HL6)), which was renewed every 30 min. HL6, shown in Table 1 (components from Sigma; pH adjusted to 7.20 with 1 M NaOH, approximately 1 ml/l), is based on recent hemolymph analyses (Pierce et al. 1999).

Electrophysiological measurements were obtained with sharp intracellular microelectrodes filled with 1.5 M KCl and 1.5 M K-acetate (50–70 MΩ). Excitatory junction potentials (EJPs) and miniature EJPs (mEJPs) were recorded using an A-M Systems (Carlsborg, WA) Neuroprobe amplifier 1600, low-pass filtered at 5 kHz, and acquired to hard disk at 20 kHz by a PCI-6024E multifunction I/O card (National Instruments, Austin TX) and Strathclyde Electrophysiology Software (WCP for Windows V3.1.4, University of Strathclyde, Strathclyde, UK).

Motor neurons were forward-filled with fluorescent indicators [Oregon Green BAPTA-1 (OGB-1), 10 kDa; Calcium Green-1, 10 and 70 kDa; Calcium Crimson, 10 kDa; Fura-2, 10 kDa; and Fluo-4, 10 kDa; Molecular Probes, Eugene OR] dissolved in water. All Ca$^{2+}$ imaging, except that on Fura-2, was performed using a BioRad 600 confocal scan-head (BioRad, Mississauga, Ontario, Canada) on a Nikon microscope (Optiphot-2) with a 40 X Nikon water-immersion objective (0.55 NA). The Argon ion laser was operated at low power and further attenuated to 0.5% transmission using neutral density filters. The pinhole was opened to its maximum aperture. A green light-emitting-diode, placed in the optical path, was set for 2 ms to mark the beginning of stimulation. Fluorescence (F) is consistently reported with background subtracted [arbitrary units (au); Figs. 3 and 4]. ΔF/F is defined as the change in F during stimulation, relative to F prior to nerve stimulation. N denotes an animal while n denotes a measurement.

Fura-2 fluorescence images were acquired through a 530 ± 35-nm band-pass filter by an intensified CCD camera (model IC-100, PTI, Princeton NJ). A filter-wheel (model 5240, Pacific Scientific, Metaltek Instruments, Raleigh NC) was used to excite the preparation with light from a mercury arc lamp alternately through 350 ± 5 nm and 385 ± 5 nm band-pass filters (Omega Optical, Brattleboro, VT). The camera and filter-wheel were mounted on a Nikon (Optiphot-2) microscope (Olympus water-immersion objective: 40×, 0.7 NA) and controlled by an Axon Instruments (Foster City CA) Digidata 2000 frame-grabber supported by AIIW 2.2 software. Equation 5 of Grynkiewicz et al. (1985) was used to calculate absolute [Ca$^{2+}$], values. Values of $R_{\text{min}}$ and $R_{\text{max}}$ were obtained in situ through bath application of 20 μM ionomycin (Calbiochem, La Jolla CA) in HL6 containing either no added Ca$^{2+}$ and 5 mM EGTA (Sigma; $R_{\text{min}}$) or 2 mM [Ca$^{2+}$]$_i$ (R$_{\text{max}}$). The value used for the dissociation constant (K$_{\text{diss}}$; 371 nM) of...
TABLE 1. Physiological solutions

<table>
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<th>Standard (mM)</th>
<th>HL3 (mM)</th>
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<td>CaCl₂</td>
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<tr>
<td>Isethionic acid (Na⁺)</td>
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<td>—</td>
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<tr>
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<tr>
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<tr>
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<td>341</td>
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BES, (N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid); HEPES, (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]); trolox (Aldrich, Milwaukee, WI); TPEN, tetrakis-(2-pyridylmethyl)ethylenediamine (Molecular Probes). * Included for optophysiology.

Fura-2 was that determined in situ for an astrocyte cell line by Petr and Wurster (1997).

RESULTS

To maintain a physiologically stable preparation long enough to allow dissection, indicator transport, and measurement, we needed to develop a suitable physiological solution. Preliminary trials showed that previous solutions employed for this preparation (Table 1) do not maintain its physiological stability for an adequate period. In the standard solution introduced by Jan and Jan (1976), vacuolation of muscle cells and unstable resting membrane potentials (RMPs) are commonly observed (Stewart et al. 1994). The more “hemolymph-like” solution (HL3) introduced by Stewart et al. (1994) produces less muscle cell vacuolation and more stable RMPs than the standard solution. Even in HL3, some vacuolation is frequently observed (Fig. 1A), indicating that the solution is not fully compatible with the preparation. More significantly, in HL3 solution, nerve excitability is often lost after 2 h, while muscle cells often exhibit destructive spontaneous contractions.

We tested the effects of hemolymph components (Pierce et al. 1999) on muscle cell vacuolation and RMP. Trehalose in combination with the principal hemolymph amino acids, at the concentrations reported by Pierce et al. (1999), eliminated vacuolation completely (Fig. 1B). Correspondingly, motor axon excitability was maintained with no change in threshold for a longer time. Axon excitability threshold (1.52 ± 0.68 V,

mean ± SD, N = 61; 0.3 ms) remained constant when stimulation amplitude was maintained below double threshold. Although the improved solution has 31% less Cl⁻ than HL3, solutions in which only [Cl⁻] was reduced produced inconsistent results for vacuolation and excitability. [K⁺] was increased from 5 to 25 mM, closer to hemolymph [K⁺] values. Thus the HL6 solution (Table 1) incorporated the amino acids, normal blood sugar trehalose, and [K⁺] of 25 mM. Glutamate, although reported in hemolymph (Pierce et al. 1999; but see Irving et al. 1979), was omitted because it interferes with synaptic transmission.

The time course of the EJP recorded in HL6 is indistinguishable from that in HL3, and EJPs summate when the nerve is stimulated at a sufficient frequency (Fig. 1C). EJP amplitude and time-course did not change appreciably for several hours. The average RMP in muscles 6 and 7 was initially 62.3 ± 5.7 mV and was still 53.1 ± 6.9 mV after 5.5 h in HL6 (41 cells, N = 6; Fig. 1D). The high RMP in this relatively high [K⁺] solution is predicted from the low [Na⁺]o ([54.7 mM], as the dependence of the muscle RMP follows the Goldman-Hodgkin-Katz equation with a ratio of 0.23 for Na⁺ to K⁺ permeability (Jan and Jan 1976). The frequency of mEJPs,
Over 40 min, and viewed with confocal microscopy (17–muscles 7, 6, 13, and 12, forward-filled with 1 mM Oregon Green BAPTA-1 nerve. Scale bar, 100 μm. 

Fig. 2. Motor neuron terminals filled with dextran-conjugated indicators. A: severed end of a hemi-segment nerve fits snugly in the loading pipette. Scale bar, 50 μm. Inset: nerve (n) held in the tip of a pipette (p), showing the tip of the dye delivery tube (t). B: pipette in place over the mid-line of a filleted larval preparation. C: same field of view as in B, observed using epi-fluorescence optics. Fluorescent indicator is visible in the lumen of the pipette and in the nerve. Scale bar, 100 μm (B and C). D: segment nerve (3) at body-wall muscles 7, 6, 13, and 12, forward-filled with 1 mM Oregon Green BAPTA-1 (OGB-1) over 40 min, and viewed with confocal microscopy (17-μm depth, z-series projection). Indicator fluorescence is clearly visible in boutons of the motor neuron terminals. Scale bar, 20 μm. E: bouton types Ib and Is on muscles 7 and 6. F: bouton types Ib, Is, and II on muscle 13. Scale bar, 20 μm in both E and F (6-μm depth projections). 

Fluorescence intensity minus background (F0 – F) occurred (Fig. 3A). Fluorescence (F) increased rapidly toward a plateau over the first 1 s of stimulation (Fig. 3B) with OGB-1 (Kd = 170 nM; Molecular Probes). The level of the plateau in Fig. 3C is approximately linearly related to the stimulation frequency from 5 to 20 Hz (Fig. 3D). Average ΔF/F at 10-Hz stimulation is 60 ± 6% (mean ± SE, N = 6). The extracellular Ca2+ concentration ([Ca2+]o) in HL6 was set at 0.5 mM, the highest level at which [Ca2+]o could be maintained without muscle contraction at 20-Hz stimulation. 

Fig. 3. Fluorescence changes in response to trains of stimulation pulses. A: 4 sequentially scanned images of type Ib boutons filled with OGB-1 on muscle 6. Each image is scanned left-to-right, 2 ms per line, and is 128 lines long (256 ms). Distance between consecutive images represents an acquisition delay (304 ms). White scan line in the second image marks the start of a 5-s train of electrical pulses (2 V, 0.3 ms) delivered to the nerve at 20 Hz. Pulses are represented by a series of arrow ticks below the images. The look-up-table, used to represent fluorescence intensity (0–255) in each pixel, is shown on the right. Scale bar, 10 μm. B: time course of the average pixel fluorescence intensity minus background (F) of the bouton enclosed by a box in the 4th image in A, during a 20-Hz stimulus train [arbitrary units (au)]. Open symbols correspond to the 4 images presented in A. C: percentage change in bouton F (ΔF/F) in response to 5-s stimulation trains of different frequencies (5, 10, 20, 30, 40 Hz; n = 3, 3, 3, 1, 1, respectively). Data for frequencies above 20 Hz could not be collected beyond the start of the trains due to muscle contraction. D: a plot of average maximum ΔF/F reached during the trains of different frequencies in C.
that Ca\(^{2+}\) builds up. The consistent height of consecutive pulses did not accumulate from one pulse to the next, \(\tau\) determined in motor neurons in the present study.

Almost all loaded preparations showed a response to a single nerve stimulus. Fast sampling by confocal line scanning (Fig. 4) revealed fast intracellular Ca\(^{2+}\) dynamics. At 2-Hz stimulation (Fig. 4, A and B), the plot of \(F\) indicates that [Ca\(^{2+}\)] does not accumulate from one pulse to the next, whereas at frequencies 10 Hz and above (Fig. 4, C and D), [Ca\(^{2+}\)] builds up. The consistent height of consecutive transients is a good indication that the calcium indicator (OGB-1) is not saturating in this [Ca\(^{2+}\)] range. F decay of OGB-1 for 200 ms following a single isolated pulse was fitted by a single exponential \(\tau = 58.8 \pm 8.4\) (SE) ms, \(N = 6\), as was \(F\) decay following the last pulse of a 1-s stimulus train at 20 Hz \(\tau = 61.8 \pm 6.6\) ms, \(N = 3\). Using the lower affinity indicator, Fluo-4 10 kDa \((K_D = 3.1\) \(\mu\)M; Kreitzer et al. 2000), \(F\) decay for 200 ms following a single pulse was 55.4 \pm 8.6\) ms (\(N = 4\)). The \(K_D\) of both indicators was established in vitro in a 100 mM KCl solution; however, the \(K_D\) of these indicators is likely to be several-fold higher in the motor neuron cytoplasm (Thomas et al. 2000). Measurements using Fluo-4 10 kDa were made in Schneider’s which provided a high [Ca\(^{2+}\)]\(_i\) (5.4 mM) while also preventing movement during high-frequency stimulation trains. To simulate locomotor activity, motor neuron terminals filled with Fluo-4 10 kDa were stimulated at 80 Hz for 2 s in the high [Ca\(^{2+}\)]\(_i\) environment of Schneider’s. \(F\) decay of Fluo-4 following the last pulse of the train was also rapid; 90.4 \(\pm\) 6.5 ms \((N = 4\), single exponential fit over 750 ms\). A curve fit to the sum of two exponentials did not provide a better description of \(F\) decay. Where high concentrations of calcium indicator are used in the loading solution (>2 mM), and to a lesser extent when loading times are excessive (>40 min), \(\tau\) measured using OGB-1 has been estimated at over 100 ms.

To verify that these techniques can be used with ratiometric imaging we used Fura-2 10 kDa dextran to estimate the resting [Ca\(^{2+}\)]. In seven preparations, the average [Ca\(^{2+}\)] was \(23 \pm 11\) (SE) nM. Preliminary data indicate that resting [Ca\(^{2+}\)] may be higher in the standard solution of Jan and Jan (1976) than in HL6, suggesting that the new solution better preserves presynaptic [Ca\(^{2+}\)], homeostasis.

**Discussion**

We have provided a reliable assay for [Ca\(^{2+}\)] in *Drosophila* larval motor neuron terminals; kinetics of signals for single stimuli and for trains; and a new physiological solution, HL6, which supports longer periods of vitality for the larval preparation, allowing the application of techniques that require longer execution times. The time-constant of Ca\(^{2+}\) clearance is rapid for both single pulses and high-frequency trains, indicating a very efficient extrusion mechanism.

The technique of forward-filling has the advantages of high sensitivity, good temporal resolution, exclusion from intracellular organelles, and stable indicator concentration. In addition, it provides the potential for loading terminals with other substances for probing synaptic function. We found that molecules as large as 70 kDa (Calcium Green-1 70 kDa dextran) can be loaded into axons. Thus other molecules such as chaperone proteins, competitive peptide fragments, and caged compounds may load successfully. This opens up a wide range of acute experiments in which intracellular proteins and reagents can be added or modified.

Kerr et al. (2000) used the GAL4-upstream activating sequence (UAS) system to drive expression of the Ca\(^{2+}\) indicator protein, cameleon, in the neurons of *Caenorhabditis elegans*. The same technique has been applied to the motor neuron terminals of larval *Drosophila* (Reiff and Schuster 2000). However, this technique cannot be used to detect responses to single pulse stimulation in single cells. Even the most recent version of cameleon indicators (Truong et al. 2001) has not demonstrated sufficient Ca\(^{2+}\) responsiveness to detect a signal from one AP in single cells.

The estimate of resting [Ca\(^{2+}\)], reported here (23 nM) is similar to that reported in *Drosophila* giant neuron cell culture (22 nM; using a cell-free Fura-2 \(K_D\) estimate of 147 nM) (Berke and Wu 2002). However, estimated [Ca\(^{2+}\)] values are very sensitive to the \(K_D\) used, and the Fura-2 \(K_D\) was not determined in motor neurons in the present study.

The plateau level of [Ca\(^{2+}\)] accumulation increases in direct proportion to the frequency of pulses (see Helmchen et al. 1996), indicating that the Ca\(^{2+}\) clearance mechanism does not saturate at these levels of Ca\(^{2+}\) influx. The rapid fluorescence decay when stimulation ceases, indicates an efficient Ca\(^{2+}\) clearance mechanism. Even after long trains of stimulation, we did not observe a fluorescence decay time-constant on the order of minutes as reported by Umbach et al. (1998).
The technique described here has been used to examine whether there is a Ca\(^{2+}\) handling defect associated with the reduction in neurotransmitter release at the motor neuron terminals in the cacophony temperature-sensitive Drosophila mutant cac\(^{ts2}\) (Kawasaki et al. 2000) at higher temperatures. There is a profound reduction in Ca\(^{2+}\) entry into boutons in response to single APs as part of the larval cac\(^{ts2}\) phenotype at higher temperatures (Macleod et al. 2001). The technique has also been applied at the motor neuron terminals of csp mutant Drosophila larvae and preliminary data (K. Dawson-Scully and G. T. Macleod, personal observations) confirm earlier observations using the AM calcium indicator loading protocol of Dawson-Scully et al. (2000).

Detection of Ca\(^{2+}\) entry into individual boutons with single APs provides the means to test hypotheses for the regulation of [Ca\(^{2+}\)]. The time-constant reported here, approximately 60 ms at 21°C, is fast relative to values for other neuron terminals at room temperature (Helmchen and Tank 1999). There are no adjacent sinks in the form of large inter-bouton connections to explain the rapid decay in terms of diffusion. We postulate that Drosophila motor neuron terminals possess a relatively large complement of Ca\(^{2+}\) extrusion molecules, consistent with their maintained high rates of impulse activity during locomotion when motor axons conduct AP volleys at \(\pm 100\) Hz, every couple of seconds (Barclay et al. 2002).

ACKNOWLEDGMENTS

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