Role of ATP-Dependent Calcium Regulation in Modulation of *Drosophila* Synaptic Thermotolerance

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Klose MK, Boulianne GL, Robertson RM, Atwood HL. Role of ATP-dependent calcium regulation in modulation of *Drosophila* synaptic thermotolerance. *J Neurophysiol* 102: 901–913, 2009. First published May 27, 2009; doi:10.1152/jn.91209.2008. Maintenance of synaptic transmission requires regulation of intracellular Ca^{2+} in presynaptic nerve terminals; loss of this regulation at elevated temperatures may cause synaptic failure. Accordingly, we examined the thermosensitivity of presynaptic calcium regulation in *Drosophila* larval neuromuscular junctions, testing for effects of disrupting calcium clearance. Motor neurons were loaded with the ratiometric Ca^{2+} indicator Fura-dextran to monitor calcium regulation as temperature increased. Block of the Na^+/Ca^{2+} exchanger or removal of extracellular Ca^{2+} prevented the normal temperature-induced increase in resting calcium. Conversely, two treatments that interfered with Ca^{2+} clearance—inactivation of the endoplasmic reticulum Ca^{2+}-ATPase with thapsigargin and inhibition of the plasma membrane Ca^{2+}-ATPase with high pH—significantly accelerated the temperature-induced rise in resting Ca^{2+} concentration and reduced the thermotolerance of synaptic transmission. Disrupting Ca^{2+}-ATPase function by interfering with energy production also facilitated the temperature-induced rise in resting $[Ca^{2+}]$ and reduced thermotolerance of synaptic transmission. Conversely, fortifying energy levels with extra intracellular ATP extended the operating temperature range of both synaptic transmission and Ca^{2+} regulation. In each of these cases, Ca^{2+} elevations evoked by an electrical stimulation of the nerve (evoked Ca^{2+} responses) failed when resting Ca^{2+} remained >e 200 nM for several minutes. Failure of synaptic function was correlated with the release of intracellular calcium stores, and we provide evidence suggesting that release from the mitochondria disrupts evoked calcium responses and synaptic transmission. Thus the thermal limit of synaptic transmission may be directly linked to the stability of ATP-dependent mechanisms that regulate intracellular ion concentrations in the nerve terminal.

INTRODUCTION

Exposure to hyperthermia increases resting calcium concentration in a variety of cell types (Cohen et al. 2007; Kiang et al. 1992). In *Drosophila,* the hyperthermia-induced loss of locomotor pattern generation and behavior correlates with the failure of synaptic function and a large increase in presynaptic resting calcium concentration (Klose et al. 2005, 2008). Treatments such as heat shock or Hsp70 expression, which thermoprotect parameters of synaptic function including quantal content (Karunanithi et al. 1999, 2002), also improve the nerve terminal's ability to maintain near-normal resting calcium concentration at high temperatures (Klose et al. 2008). This suggests a direct relationship between presynaptic mechanisms

of calcium regulation and the thermotolerance of synaptic function. Loss of neuromuscular transmission at high temperature is not the result of unresponsive Na^+ channels, nor is it the result of postsynaptic glutamate receptor inactivation; these observations further support the primacy of presynaptic mechanisms (Klose et al. 2005, 2008).

The loss of normal cytosolic calcium regulation is common during stress-induced pathologies (Bickler and Buck 1998; Buck and Parmenter 2006; Stys 2004). Pathological elevations in free calcium activate destructive enzymatic activity resulting in cytoskeletal disruption, DNA fragmentation, and organelle damage (Budd 1998; Orrenius et al. 1989). Thus preventing large increases in resting intracellular calcium ion concentration ($[Ca^{2+}]_i$) enhances cellular survival. However, what is responsible for the temperature-induced rise in calcium and what role it may play in disrupting evoked calcium responses and synaptic transmission is unclear.

In the present study, we examined calcium regulation in nerve terminals at rest and during stimulation to address the importance of processes that regulate intracellular Ca^{2+} and thermosensitivity of synaptic function. The neuromuscular junction of the fruit fly, *Drosophila melanogaster* provides a synaptic preparation in which $[Ca^{2+}]_i$ can be measured at rest and during stimulus trains using the ratiometric fluorophore Fura-dextran (Macleod et al. 2002, 2003) and in which both genetic and pharmacological interventions can be achieved. Calcium entering through voltage-gated calcium channels is rapidly sequestered by organelles or cleared out of the nerve terminal at the surface membrane. We functionally disrupted these calcium clearance mechanisms one at a time, using pharmacological or genetic procedures to assess their effects on calcium regulation at elevated temperatures The results support the hypothesis that ATP-dependent calcium clearance mechanisms are major determinants of the thermotolerance of Ca^{2+} responses evoked by nerve activity and of sustained synaptic transmission.

METHODS

Fly stocks

All the flies were raised on standard cornmeal medium at 25°C and 60–70% humidity. Early wandering third-instar larvae were selected for the experiments. *Drosophila* lines used were Canton-S unless otherwise stated.

The *dmiro* mutant flies and their controls, P[ry+,FRT] 83B,ry−, were obtained from Dr. Konrad Zinsmaier (University of Arizona). These flies were generated through ethylmethane sulfonate (EMS) mutagenesis and screened for altered phototaxis (Guo et al. 2005). In these mutants, proper distribution of mitochondria is disrupted by a

mutation in mitochondrial Rho-GTPase preventing transport into nerve terminals where synaptic transmission is initiated. Mutant lines were kept over a *TM6 Tb* balancer chromosome.

Experimental procedures

For physiological experiments, dissections were carried out in Schneider's insect medium (Sigma-Aldrich) to minimize contractions and reduce the chance of damage during dissection. The larvae were opened by a mid-dorsal incision, and the internal organs were then removed, leaving the nervous system and body wall muscles intact. Physiological recordings were obtained from preparations that were continuously superfused with HL6 solution (Macleod et al. 2002) containing 0.5 mM Ca^{2+}. HL6 contained (mM) 0.5 $CaCl_2$, 15.0 $MgCl_2$, 24.8 KCl, 23.7 NaCl, 10.0 $NaHCO_3$, 20.0 isethionic acid, 5.0 *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), 80.0 trehalose·$2H_2O$, 5.7 L-alanine, 2.0 L-arginine·HCl, 14.5 glycine, 11.0 L-hisatidine, 1.7 L-methionine, 13.0 L-proline, 2.3 L-serine, 2.5 L-threonine, 1.4 L-tyrosine, 1.0 L-valine, 1.0 Trolox, and 0.0001 *N,N,N′,N′*-tetrakis-(2-pyridyl-methyl)-ethylenediamine (TPEN). L-glutamate (7 mM) was added to the solution to desensitize glutamate receptors to prevent muscle contractions during stimulation and Ca^{2+} imaging. (Macleod et al. 2002).

To identify the mechanisms regulating synaptic thermotolerance, several pharmacological manipulations were employed. To disrupt the plasma membrane Ca^{2+}-ATPase, we replaced BES buffer with Tris and the pH was elevated to 8.8 with NaOH. The sodium/Ca^{2+} exchanger was disrupted by replacing sodium chloride with *N*-methyl-D-glucosamine (Sigma-Aldrich) (Lnenicka et al. 2006). In Ca^{2+}-free experiments, calcium was removed and 1 mM EGTA was added to the saline solution. In one set of experiments, we tested the effects of high pH using both HL6 and HL3 solution (Stewart et al. 1994).

In experiments conducted to test thermosensitivity, temperature was increased in a stepwise manner in increments of 2°C. Recordings were taken after 4 min at each individual temperature so that each preparation was subjected to the same stress dose (Klose et al. 2004). For the calcium studies that employed Fura-dextran, images were taken once every 5 s, during 20 s of rest, during 20 s of 30-Hz stimulation and during 20 s immediately following the cessation of stimulation. Electrophysiological experiments were run using the same protocol used in the calcium imaging experiments with two exceptions: no glutamate was put in the saline and in between bouts of 30-Hz stimulation, the nerve was stimulated at 0.3 Hz to determine more precisely the temperature of failure of synaptic transmission.

A fast or steep temperature ramp results in much higher failure temperatures than obtained with slower temperature ramps (Gerner 1987; Klose et al. 2004). A very high temperature exposure for a short duration can have the same ill effects as a less extreme temperature over a longer period of time. Thus temperature ramp rate was consistent across all experiments so that the failure temperature could be used as a reliable measure of thermotolerance.

Calcium measurements

Measurements of Ca^{2+} concentration in type 1_b (large, tonic) nerve terminals were obtained by loading the calcium indicator Fura-dextran (10 kDa; Molecular Probes) into the cut end of the motor axon, as described in Macleod et al. (2002).

Fluorescence from the nerve terminals of Fura-dextran-loaded neurons was detected with an intensified CCD camera (PTI, model IC-100; Princeton, NJ) connected to a Lightning 2000 frame grabber controlled by Axon Imaging Workbench 2.2 (AIW 2.2; both from Axon Instruments, Union City, CA). Emitted light was collected by an Olympus water-immersion objective (×40, 0.7 NA) through a 530 ± 35-nm band-pass filter, both mounted on an upright Nikon (Optiphot-2) microscope. The loaded neurons were excited with light from a mercury arc lamp alternately through 350 ± 5 and 385 ± 5 nm

band-pass filters on a rapidly switching filter wheel (Omega Optical, Brattleboro, VT).

Calibration experiments were conducted to obtain the maximum (R_{max}) and minimum (R_{min}) fluorescence values for the preparation in the experimental chamber. These values were used to calculate the values for calcium recorded with the Fura-dextran (Grynkiewicz et al. 1985). For R_{max}, HL6 containing 10 mM Ca^{2+} and 1 μM ionomycin was employed. Then the solution was replaced with HL6 containing no calcium, 10 mM EGTA, and 1 μM ionomycin to obtain R_{min}. The value for K_d was taken as 865 nM following previous work by Macleod et al. (2002) and Bronk et al. (2005).

ImageJ software (http://rsb.info.nih.gov/ij/) was used to convert PIC image files to TIFF format, allowing measurement of average pixel value within selected image regions. A region not containing any calcium indicator fluorescence or autofluourescence was randomly selected beside each fluorescent nerve terminal and the average pixel intensity was measured in this region to give a background value.

Stimulation protocol

In both electrophysiological and calcium-imaging experiments, nerves were stimulated by a square pulse with a duration of 1.9 ms and just-suprathreshold stimulus intensity. Occasionally at moderate temperatures, negative pressure applied to the nerve or a small increase in stimulus intensity (0.5 V) was needed to restore the evoked calcium response.

In experiments that had no sodium in the physiological saline, the stimulus intensity was increased to as much as 100 V to electrotonically stimulate voltage-activated calcium channels in the nerve terminal. Electrotonic stimulation has been used successfully at the *Drosophila* neuromuscular junction to directly recruit voltage-activated calcium channels independently of action potentials (Bronk et al. 2001; Klose et al. 2008). On failure of synaptic responses, electrotonic stimulation was used to bypass Na^+ channels and directly stimulate Ca^{2+} channels. Neither the excitatory junctional potential nor the evoked Ca^{2+} response recovered with electrotonic stimulation in any of the experiments.

Pharmacological experiments

Thapsigargin (Sigma) was solubilized in 0.01% DMSO. To get reliable effects, preparations were subjected to a 1-h bath application of 20 μM thapsigargin and then superfused with saline containing 2 μM thapsigargin as a precaution against washout. Controls contained 0.01% DMSO in the physiological solution. To release calcium from mitochondrial stores, 20 μM veratridine was solubilized in 0.01% DMSO and applied to nerve terminals, whereas 20 mM caffeine (Sigma) was used to release calcium from the endoplasmic reticulum (Lnenicka et al. 2006; Shakiryanova et al. 2007). Ouabain (30 μM, Sigma) was used to inhibit the Na^+-K^+ ATPase (Torrie et al. 2004). The protonophore carbonyl cyanide m-chloro phenyl hydrazone (CCCP) (Sigma) was used to disrupt mitochondrial function. The Na^+/Ca^{2+} exchanger was disrupted by replacing sodium ions with *N*-methyl-D-glucosamine (Sigma), while the plasma membrane Ca^{2+}-ATPase was disrupted by elevating saline pH to 8.8 by substituting Tris buffer for the buffer normally present in the HL6 solution BES (Lnenicka et al. 2006).

Preliminary experiments at room temperature revealed that both CCCP and veratridine elevated resting calcium levels and prevented evoked responses within minutes of exposure.

None of the other pharmacological agents on their own disrupted evoked responses or elevated the resting calcium substantially within the first hour of application. Because the temperature ramp thermotolerance test took <45 min, the effects observed are the combined result of the temperature stress and the pharmacologically disrupted mechanism. Prolonged exposure of the preparation to iodoacetate at room temperature did, however, lead to an elevation in resting

calcium concentration and disruption of the evoked response, but only after >45 min of exposure. Other preliminary data showed that the time-dependent effects of iodoacetate were attenuated by exogenous ATP loaded into nerve terminals. The protective effects of the ATP lasted well over 90 min.

To test for the effect of prolonged elevation of resting calcium concentration on stimulus-evoked calcium response fidelity independently of elevated temperature, we placed a macropatch electrode (Wong et al. 1999) filled with ionomycin over a nerve terminal bouton loaded with Fura-dextran. Images were taken as described in the preceding text. Nerve terminal calcium concentration was assessed simultaneously in both a terminal exposed to ionomycin and a terminal distal to the macropatch electrode. Control experiments were carried out to examine the effect of imaging through a macropatch electrode and no difference in ratiometric measurements was detected, as previously shown for crayfish nerve terminals (Millar et al. 2005; Ravin et al. 1997).

Statistical analysis

Significance of $P < 0.05$ (unless otherwise stated), was assessed with a t-test, or two-way ANOVA with the appropriate post hoc test (Holm-Sidak) where applicable. All values are reported as means $\pm$ SE.

RESULTS

We investigated the role of presynaptic calcium clearance mechanisms in synaptic thermotolerance. Nerve-evoked Ca^{2+} transients were monitored as an indicator of presynaptic competence in Ca^{2+} homeostasis during normal activity.

Steady-state free calcium concentration was measured using Fura-dextran in the type 1b nerve terminals on muscles 6 and 7 (Fig. 1A). Rapid switching between 340 and 380 nm excitation allowed ratiometric calculation of intra-terminal $[Ca^{2+}]$. At each temperature, recordings were taken three times every 5 s: 20 s before, 20 s during, and 20 s after 30-Hz stimulation. The $[Ca^{2+}]_r$ increased dramatically as temperature was raised from 39 to 41°C. The amplitude of the stimulus-evoked Ca^{2+} response was compromised at 41°C and $[Ca^{2+}]_r$ and did not return to peristimulus levels during the 20-s post stimulus recording period as it did at lower temperatures (Fig. 1B). At 43°C, the $[Ca^{2+}]_r$ remained >200 nM, and the nerve terminals became unresponsive to stimuli.

The failure temperature of synaptic transmission was assessed by determining the temperature at which nerve stimulation failed to excite a postsynaptic potential during a temperature ramp (Fig. 1C). Previous studies using this procedure (Karunanithi et al. 1999; Klose et al. 2008) have thoroughly described the effects of hyperthermia on excitatory junction potentials and currents (EJPs and EJCs). These studies indicated that presynaptic mechanisms limit thermotolerance of synaptic function. As temperature increases, synaptic responses decrease in amplitude (Fig. 1C). Prior to the failure of synaptic transmission, a large increase in spontaneous transmitter release is observed (Fig. 1D). We used these indicators as a measure of synaptic competence.

Role of ATP-dependent Ca^{2+} pumps in thermotolerance of calcium regulation

Calcium can be actively pumped from the cytosol into the extracellular compartment through the plasma membrane Ca^{2+}-ATPase (PMCA) or into the endoplasmic reticulum
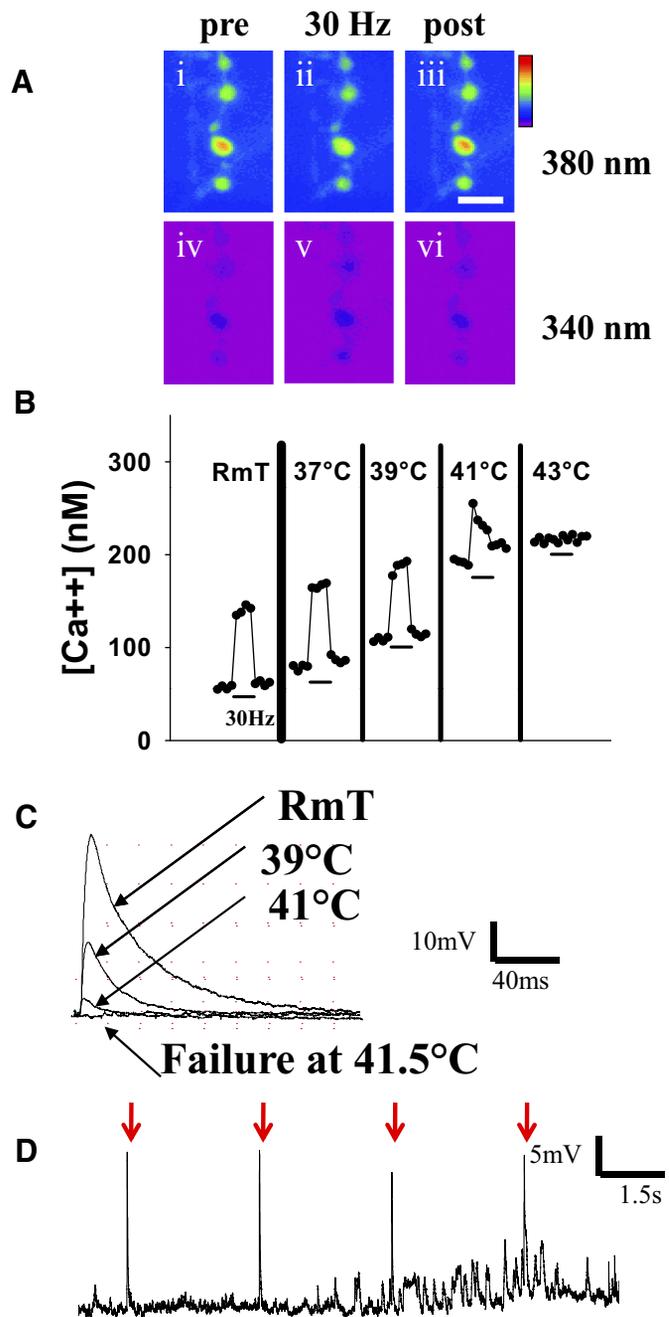

FIG. 1. A: effects of raising temperature on resting Ca^{2+} and nerve-evoked Ca^{2+} responses. Fura-dextran was forward-loaded into nerve terminals and imaged through a 510-nm LP emission filter using 380 nm (i–iii) and 340 nm (iv–vi) excitation wavelengths. Images were taken at rest before stimulation (i and iv), during 30-Hz stimulation of the nerve (ii and v), and at rest following stimulation (iiii–vi). Bar = 10 μm. B: nerve terminal calcium regulation during an increasing temperature ramp. Temperature was elevated by 2°C and then held for four minutes before each recording. Steady-state $[Ca^{2+}]$ was measured every 5 s at rest, during 20 s of 30-Hz stimulation (horizontal bar), and following stimulation. Recordings at room temperature, 37, 39, 41, and 43°C show deterioration of calcium dynamics at 41°C and disappearance of evoked calcium excursions at 43°C. C: intracellular recordings from abdominal muscle 6 in the 3rd segment during an increasing temperature ramp until failure of synaptic transmission. Excitatory junctional potentials (EJPs) recorded at room temperature, 39.5°C and 41.0, and failure of EJPs occurred at 41.5°C. D: increase in frequency of spontaneous release from 0.5 to 6 Hz precedes failure of excitatory junctional potentials by ~3 min in this preparation. Red arrows indicate EJPs.

through the (SERCA). Disruption of either calcium clearance mechanism facilitated the temperature-dependent rise in $[Ca^{2+}]_r$ and reduced the upper temperature limit at which calcium responses could be evoked by stimulation (Fig. 2).

High pH was used to disrupt PMCA function (Lnenicka et al. 2006) to investigate its role in synaptic thermotolerance. We increased pH by substituting BES with Tris buffer. At room temperature, high pH (8.8) slightly increased evoked calcium response amplitude (stimulus concentration – resting concentration) from 95.8 ± 9.8 nM in controls (n = 16) to

FIG. 2. Disruption of ATP-dependent calcium clearing mechanisms reduces the themotolerance of presynaptic calcium regulation. *A*: disruption of plasma membrane calcium-ATPase by high pH reduced the themotolerance of presynaptic calcium regulation. *i*: a rise in extracellular pH to 8.8 elevated the evoked calcium response amplitude from 90.8 ± 9.8 nM (*n* = 16) to 115.3 ± 4.7 nM at room temperature (RmT). High pH facilitated the temperature-induced rise of $[Ca^{2+}]_r$. *ii*: at 37°C, the $[Ca^{2+}]_r$ was 194.6 ± 25.4 nM, which was significantly greater than in control boutons, which were 95.6 ± 4.1 nM. Calcium-responses of small amplitude could only be evoked in high pH preparations with electrotonic stimulation. *iii*: at 39°C, the $[Ca^{2+}]_r$ in high pH preparations was 199.5 ± 11.2 nM, significantly higher than in controls, 142.9 ± 6.9 nM, and evoked responses were completely disrupted, unlike in the controls which still responded. *B*: dyisruption of sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) function by thapsigargin reduces nerve terminal calcium thermotolerance. *i*: at room temperature, application of thapsigargin (Thaps; *n* = 8) significantly enhanced $[Ca^{2+}]_r$ from 38.1 ± 3.9 nM in control to 61.5 ± 5.0 nM in treated terminals. Thapsigargin also significantly increased response amplitude at RmT from control levels of 86.8 ± 7.5 nM to 144.3 ± 15.4 nM. At RmT, the entire calcium load could be removed within 5 s. *ii*: the $[Ca^{2+}]_r$ and response amplitude remained significantly higher in thapsigargin-treated preparations at 37°C. *iii*: the response amplitude was significantly diminished at 39°C.

115.3 ± 4.7 nM (*n* = 6; Fig. 2*A*). High pH facilitated the temperature-induced rise in $[Ca^{2+}]_r$ (Fig. 2*A, i–iii*). Evoked calcium responses began to fail in some preparations at 37°C (Fig. 2*Aii*). At 39°C, the $[Ca^{2+}]_r$ in terminals exposed to high pH was 199.5 ± 11.2 nM and electrotonic stimulation could not evoke a calcium response (Fig. 2*Aiii*). Published results have reported that high pH saline doubles evoked calcium response amplitudes at room temperature (Lnenicka et al. 2006). To reconcile the apparent differences, we repeated the room temperature experiments and found that the differences could be attributed to the HL3 solution used in the previous study. The amplitude of calcium responses increased by $18.8 \pm 6.0\%$ (*n* = 16) when pH was elevated in HL6 solution and by $92.2 \pm 8.3\%$ in HL3 (*n* = 4). The larger increase in the HL3 solution agreed with the previous report of Lnenicka et al. (2006), in which the HL3 solution was also used.

Failure temperature of synaptic transmission was significantly reduced from $41.0 \pm 0.8°C$ (*n* = 7) in controls to $35.1 \pm 0.7°C$ (*n* = 4) in preparations treated with high pH (Table 1). This correlated well with the reduced temperature at which evoked presynaptic calcium responses failed in terminals also exposed to high pH.

To determine the importance of the SERCA pump in maintaining synaptic function during a hyperthermic stress, we pharmacologically blocked its function using 2 μM thapsigargin. At room temperature, both $[Ca^{2+}]_r$ and stimulus-evoked calcium concentration ($[Ca^{2+}]_s$) were significantly elevated in treated terminals (Fig. 2*B*). Thapsigargin has been shown to increase the amplitude of calcium responses but only at the onset of stimulation, before calcium-induced calcium release depletes the ER stores (Kuromi and Kidokoro 2002). Thapsigargin has also been shown to slow calcium clearance from these nerve terminals (Lnenicka et al. 2006). At 37°C, $[Ca^{2+}]_r$ and $[Ca^{2+}]_s$ in thapsigargin-treated terminals were significantly elevated. However, in contrast with the situation at room temperature, not all terminals were able to sustain a calcium response for the duration of the stimulation (Fig. 2*Bii*). As temperature increased, so too did the $[Ca^{2+}]_r$, with a dramatic increase of $[Ca^{2+}]_r$ in treated terminals from 129.7 ± 17.4 nM at 37°C to 273.7 ± 39.4 nM (*n* = 8) at 39°C. This significant elevation of $[Ca^{2+}]_r$ coincided with a dramatic decline in calcium response amplitude (Fig. 2*Biii*), In fact, only two of the seven preparations responded to stimulation. The temperature at which synaptic transmission failed was significantly reduced from $41.5 \pm 0.3°C$ (*n* = 7) in control terminals exposed to physiological saline containing only DMSO to $38.5 \pm 0.6°C$ (*n* = 7) in terminals exposed to physiological saline containing DMSO and thapsigargin (Table 1). This also correlated well with the loss of evoked presynaptic calcium responses in thapsigargin-treated terminals. In each case, pharmacological disruption of ATP-dependent calcium pumps accelerated the hyperthermia-induced rise in $[Ca^{2+}]_r$ and reduced the thermotolerance of both evoked presynaptic calcium responses and postsynaptic potentials. This suggests that ATP-dependent mechanisms limit the thermotolerance level of calcium regulation.

Next we directly tested the role of ATP in thermotolerance of nerve terminal calcium regulation. In one set of experiments, glycolysis was disrupted with iodoacetate to decrease ATP levels, whereas in another set of experiments, ATP levels were increased by forward loading exogenous ATP into nerve ter-
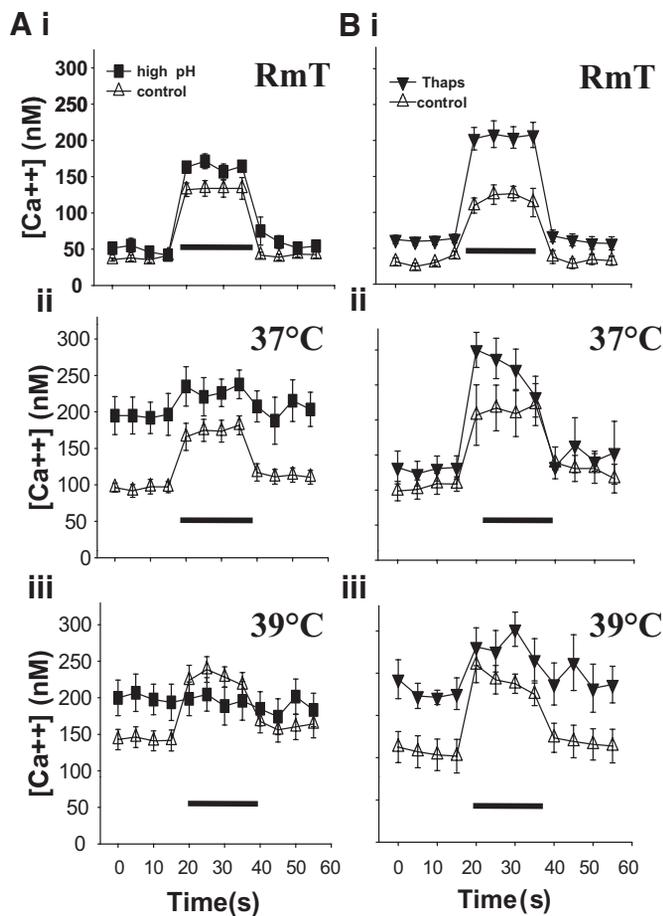
TABLE 1. *Electrophysiological characterization of synaptic function*

Test	Target	Failure Temperature of Test EJPs	Failure Temperature of Control EJPs	Change in Failure Temperature	Increase in Spontaneous Release Above 10Hz Prior to Synaptic Failure	Loss of Evoked Ca^{2-} Response Coincides With Synaptic Failure
High pH	PMCA	$35.1 \pm 0.7°C$ (4)	$41.0 \pm 0.8°C$ (7)	-5.9	Yes	Yes
Thapsigargin	SERCA	$38.5 \pm 0.6°C$ (7)	$41.5 \pm 0.3°C$ (7)	-3.0	Yes	Yes
Iodoacetate	Glycolysis	$36.4 \pm 0.3°C$ (3)	$41.0 \pm 0.8°C$ (7)	-4.6	Yes	Yes
Exogenous ATP	ATP	$43.8 \pm 0.6°C$ (6)	$41.0 \pm 0.8°C$ (7)	$+2.8$	Yes	Yes
Low Na+	Na+/Ca2+ exchanger	$33.8 \pm 0.6°C$ (4)	$40.1 \pm 1.8°C$ (4)	-6.3	Yes	Yes
Ouabain	Na+/Ca2+ exchanger	$36.9 \pm 0.3°C$ (5)	$41.0 \pm 0.8°C$ (7)	-4.1	Yes	Yes
dmiro	Nerve terminal mitochondria	$35.6 \pm 1.2°C$ (6)	$41.8 \pm 0.2°C$ (6)	-6.2	Yes	No
CCCP	Mitochondria	RmT	$41.0 \pm 0.8°C$ (7)	-20.0	Yes	Yes

Excitatory junctional potentials (EJPs) were recorded during a progressive step-wise temperature ramp and the temperature at which nerve stimulation fails to excite a post synaptic potential was assessed. Average failure temperatures are listed comparing test and control preparations as well as the difference in temperature between them. The appearance or lack of appearance of an abrupt increase in spontaneous transmitter release prior to failure of synaptic transmission was also documented. *n* values are in parentheses. PMCA, plasma membrane Ca^{2+}-ATPase; SERCA, sarco-endoplasmic reticulum Ca^{2+}-ATPase; CCCP, carbonyl cyanide m-chloro phenyl hydrazone; RMT, room temperature.

minals (Verstreken et al. 2005). Disruption of glycolysis with iodoacetate facilitated the rise in $[Ca^{2+}]_r$ (Fig. 3*A, i–iv*) and reduced the thermotolerance of evoked calcium responses (Fig. 3*Aiv*). $[Ca^{2+}]$ in iodoacetate-exposed preparations was not different from controls at room temperature (RmT). However, $[Ca^{2+}]_r$ at 37°C was 196.8 ± 10.2 nM ($n = 5$), significantly higher than controls in which $[Ca^{2+}]_r$ was 95.6 ± 4.1 nM ($n = 16$), and stimulation could not evoke a calcium response. Failure temperature of synaptic transmission was significantly reduced from $41.0 \pm 0.8°C$ ($n = 7$) in controls to $36.4 \pm 0.3°C$ ($n = 3$) in iodoacetate-treated preparations (Table 1). This correlated well with the reduced failure temperature of evoked presynaptic calcium responses in similarly treated preparations.

Fortifying ATP levels by forward loading it into terminals protected both $[Ca^{2+}]_r$ (Fig. 3*B, ii–iv*) and evoked calcium responses (Fig. 3*Biv*) at high temperatures. At 41°C, $[Ca^{2+}]_r$ in ATP-loaded terminals was 122.4 ± 5.9 nM ($n = 8$); in controls, $[Ca^{2+}]_r$ was 192.2 ± 11.3 nM ($n = 16$). At 43°C, $[Ca^{2+}]_r$ in ATP-loaded terminals was 135.9 ± 6.3 nM, and stimulation could evoke a response, whereas in controls, $[Ca^{2+}]$ was 227.0 ± 12.8 nM and responses could not be evoked. Failure temperature of synaptic transmission was significantly elevated from $41.0 \pm 0.8°C$ ($n = 7$) in controls to $43.8 \pm 0.6°C$ ($n = 6$) in ATP-fortified preparations (Table 1). This enhancement in thermoprotection paralleled the elevated failure temperature of evoked presynaptic calcium responses in similarly treated terminals. Thus exogenously loaded ATP enhanced the thermotolerance of resting calcium levels, the fidelity of evoked calcium responses, and the fidelity of synaptic transmission, while iodoacetate, which disrupts glycolytic ATP production, reduced the thermotolerance of each parameter.

Effect of prolonged calcium elevation on the fidelity of stimulus-evoked Ca^{2+} responses

Loss of evoked calcium responses occurred in each treatment when $[Ca^{2+}]_r$ was above ~200 nM regardless of temperature (Figs. 1–4*A*). However, elevation of $[Ca^{2+}]_r$ >200 nM for 20 s with 100-Hz stimulation at room temperature or 30-Hz stimulation at 39°C can be successfully repeated (Fig.

4*A*). Thus chronic elevation of calcium may be necessary to disrupt evoked calcium responses. To test this possibility, we used a calcium-specific ionophore to examine the effects of chronic elevation of $[Ca^{2+}]_r$ in single boutons on the fidelity of calcium responses at room temperature. Focal application of the ionophore to the nerve terminal was used since bath application of ionomycin elevated calcium levels in muscle as well, leading to increased tonus and muscle twitching which interfered with imaging. A macropatch electrode filled with HL6 containing ionomycin was applied to a small region of the nerve terminal without affecting the muscle. Ionomycin (0.5 μM) caused a progressive increase in $[Ca^{2+}]_r$ (Fig. 4*Bi*). Stimulus-evoked calcium responses could still be elicited when $[Ca^{2+}]_r$ was between 100 and 200 nM (10 min of application; Fig. 4*Bii*). However, when the $[Ca^{2+}]_r$ was >200 nM (30 min of application), responses could no longer be stimulated even when stimulation intensity was substantially increased in an attempt to electrotonically recruit voltage-activated calcium channels. In remote terminals of the same preparation not exposed to the ionophore, evoked calcium responses were still observed. Thus $[Ca^{2+}]_r$ of 200 nM for several minutes is sufficient to disrupt stimulus-evoked Ca^{2+} responses. Whether the hyperthermia-induced elevation in calcium results from entry of extracellular calcium or from release of internal stores is unclear.

Disruption of non-ATP-dependent Ca^2 clearance mechanisms in thermotolerance

In addition to ATP-dependent mechanisms, free ionic calcium can be cleared from the cytosol in a non-ATP-dependent manner through sequestration into the mitochondria (Guo et al. 2005) and by removal through the Na^+/Ca^{2+} exchanger into the extracellular matrix.

Replacement of extracellular Na^+ with *N*-methyl-D-glucamine (NMDG) was used to disrupt the Na^+/Ca^{2+} exchanger. Because the exchanger typically brings Na^+ into the cytosol in exchange for Ca^{2+}, we hypothesized that disrupting the exchanger would facilitate the temperature-induced rise in calcium. However, following NMDG application, the typical elevation in $[Ca^{2+}]_r$ was prevented (Fig. 5*A, i–iii*). This suggests that under normal conditions, the Na^+/Ca^{2+} exchanger reverses function at high temperature, causing an elevation of
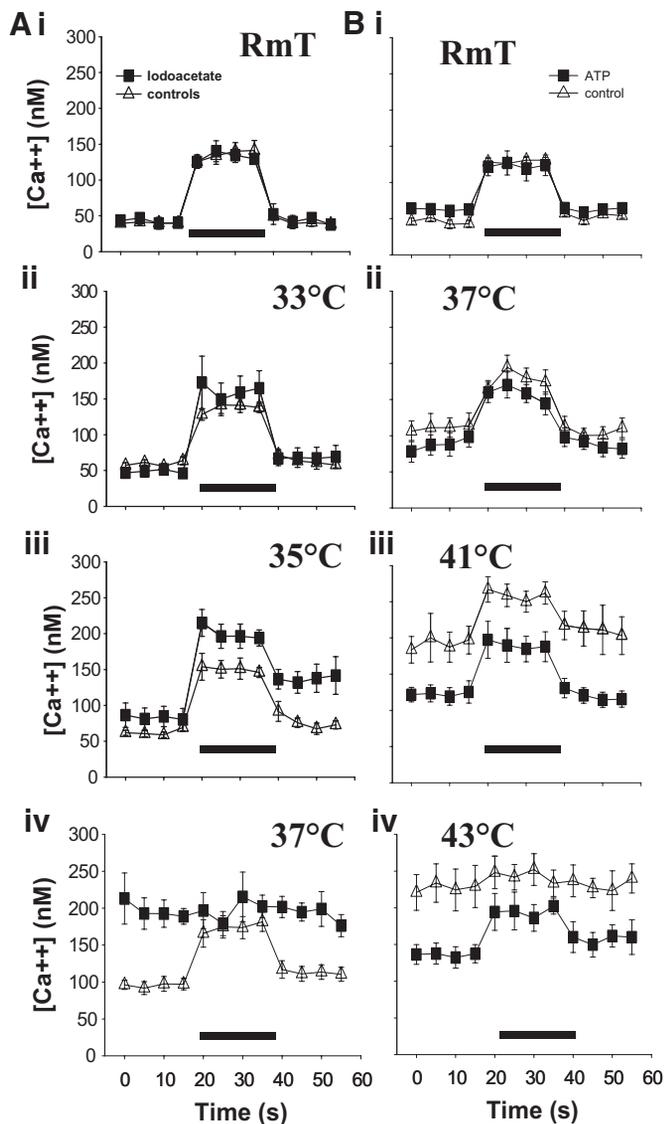
FIG. 3. Manipulation of ATP levels modulates thermotolerance of nerve terminal calcium regulation. *A*: disrupting glycolysis with iodoacetate facilitated the temperature-induced disruption of calcium regulation, reducing thermotolerance. *i*: at RmT, iodoacetate had no effect on $[Ca^{2+}]_r$ or response amplitude. *ii*: at 33°C, Ca^{2+} response amplitude is significantly larger in treated terminals. *iii*: at 35°C, both $[Ca^{2+}]_r$ and Ca^{2+} response amplitude are significantly larger in treated terminals, and note the incomplete recovery of $[Ca^{2+}]_r$ following stimulation. *iv*: at 37°C, $[Ca^{2+}]_r$ was significantly elevated, and the ability to evoked a response with stimulation was lost. *B*: forward loaded exogenous ATP thermoprotected calcium regulation. *i*: at RmT and *ii*: at 37°C, $[Ca^{2+}]_r$ and response amplitudes are similar. *iii*: at 41°C, $[Ca^{2+}]_r$ is significantly lower and Ca^{2+} response amplitudes are significantly larger in treated terminals. *Iv*: at 43°C, ATP loaded terminals maintained significantly lower $[Ca^{2+}]_r$ and are still able to respond to stimulation, unlike control terminals.

$[Ca^{2+}]_r$. At 35°C, evoked calcium responses were disrupted in spite of the relatively small rise in $[Ca^{2+}]_r$ above controls to 92.3 ± 6.5 nM ($n = 6$; Fig. 5*Aii*). In all other treatments, evoked responses were disrupted when $[Ca^{2+}]_r$ was ~200 nM. The observed small rise in $[Ca^{2+}]_r$ could result from partial function of the Na^+/Ca^{2+} exchanger pumping extracellular calcium into the cytosol or from the release of internal calcium stores. Failure temperature of synaptic transmission was significantly reduced from 40.1 ± 1.8°C ($n = 4$) in controls to 33.8 ± 0.6°C ($n = 4$)
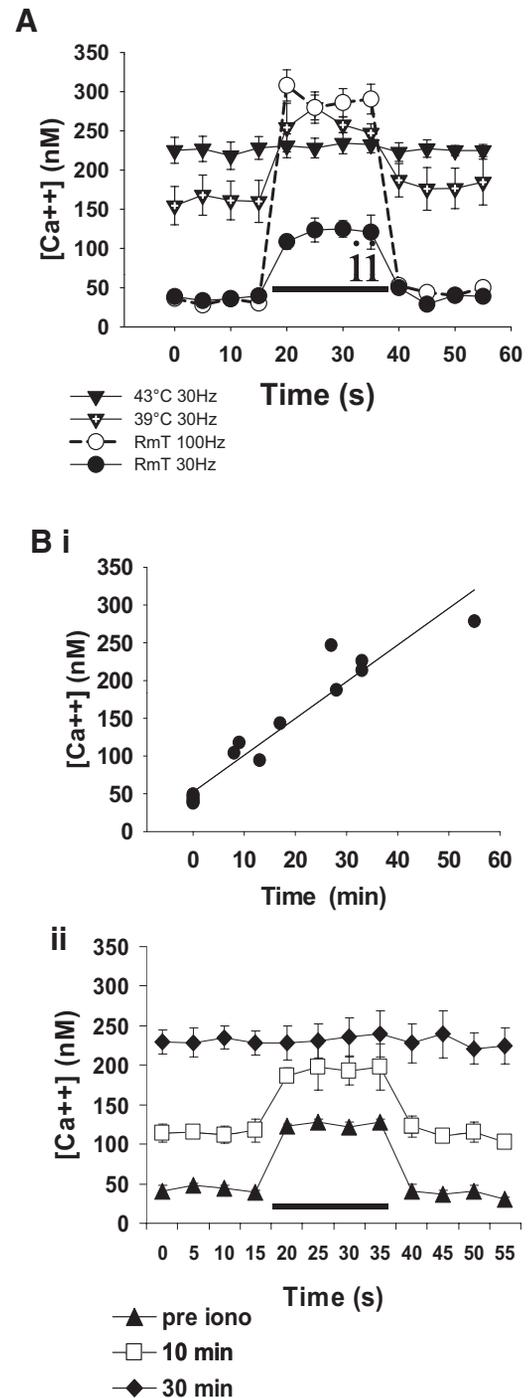
FIG. 4. Prolonged elevation of resting calcium concentration disrupts the stimulus-evoked calcium response. *A*: evoked calcium responses were successfully maintained for 20 s above 200 nM at RmT with 100-Hz stimulation and at 39°C with 30-Hz stimulation and also recovered successfully. When $[Ca^{2+}]_r$ surpassed 200 nM at 43°C, evoked responses failed. *B, i*: 0.5 μM ionomycin was loaded into a macropatch electrode, which was then placed onto a nerve terminal allowing recordings from single nerve terminals. Scatter plot with a line of best fit shows that ionomycin application resulted in a progressive increase in resting calcium concentration. *ii*: within 10 min of the application of 0.5 μM ionomycin, the $[Ca^{2+}]_r$ was elevated to 115.0 ± 10.6 nM ($n = 4$), but stimulation still yielded a response. Following 30 min of ionomycin the $[Ca^{2+}]_r$ was elevated to ~200 nM and stimulation would not yield a response.

in experiments using physiological solution with no sodium (Table 1). This reduced failure temperature paralleled the reduced temperature at which evoked presynaptic calcium responses could no longer be evoked in preparations exposed to the same conditions.

To shed further light on the source of calcium accumulation in the cytosol at high temperatures, we removed calcium from the saline and replaced it with 1 mM EGTA. Removal of Ca^{2+} from the saline had no effect on resting intracellular calcium levels at room temperature (Fig. 5Bi); however, the temperature-induced rise in resting $[Ca^{2+}]$ was attenuated (Fig. 5B, i–iii).

At 39°C, the $[Ca^{2+}]_r$ was 67.7 ± 3.4 nM without extracellular calcium in the saline. This is less than half the magnitude measured in controls with Ca^{2+} in the saline for the entire duration of the temperature ramp. When extracellular Ca^{2+} was restored at 39°C, evoked calcium responses returned with an amplitude of 87.9 ± 11.5 nM, which was not different from controls, 87.3 ± 6.7 nM (Fig. 5Bii). When the temperature was raised to 41°C in preparations that had calcium previously restored at 39°C, the $[Ca^{2+}]$ before, during, and after stimulation was not different from the controls that were bathed in Ca^{2+} throughout the entire experiment. At 41°C, in preparations without extracellular calcium, the $[Ca^{2+}]_r$ was 91.6 ± 3.3 nM, which was significantly higher than resting levels at 39°C in the same calcium-free solution. This significant rise in resting calcium from 39 to 41°C must come from internal sources since no calcium is available from external sources (Fig. 5$Biii$). Interestingly, when calcium was restored to the saline at this temperature, evoked responses could not be recovered in any preparations.

Thus under hyperthermic conditions, the Na^+/Ca^{2+} exchanger may reverse function and extrude accumulating sodium, thereby causing the entry of extracellular calcium into the cytosol and its accumulation.

To examine the effects of elevated cytosolic sodium on calcium regulation, we blocked the Na^+-K^+ ATPase with ouabain, which elevates Na^+ levels (Torrie et al. 2004). At room temperature, ouabain had no effect on resting or evoked calcium levels (Fig. 5Ci). However, at 37°C, $[Ca^{2+}]_r$ was significantly above control, and calcium responses could not be evoked (Fig. 5Cii). This reveals that elevating intracellular sodium accelerates hyperthermia-induced rise in cytosolic calcium and disruption of evoked responses, further supporting the hypothesis that the Na^+/Ca^{2+} exchanger contributes to the hyperthermia-induced misregulation of ionic homeostasis, which may ultimately lead to the disruption of both evoked calcium response and EJPs.
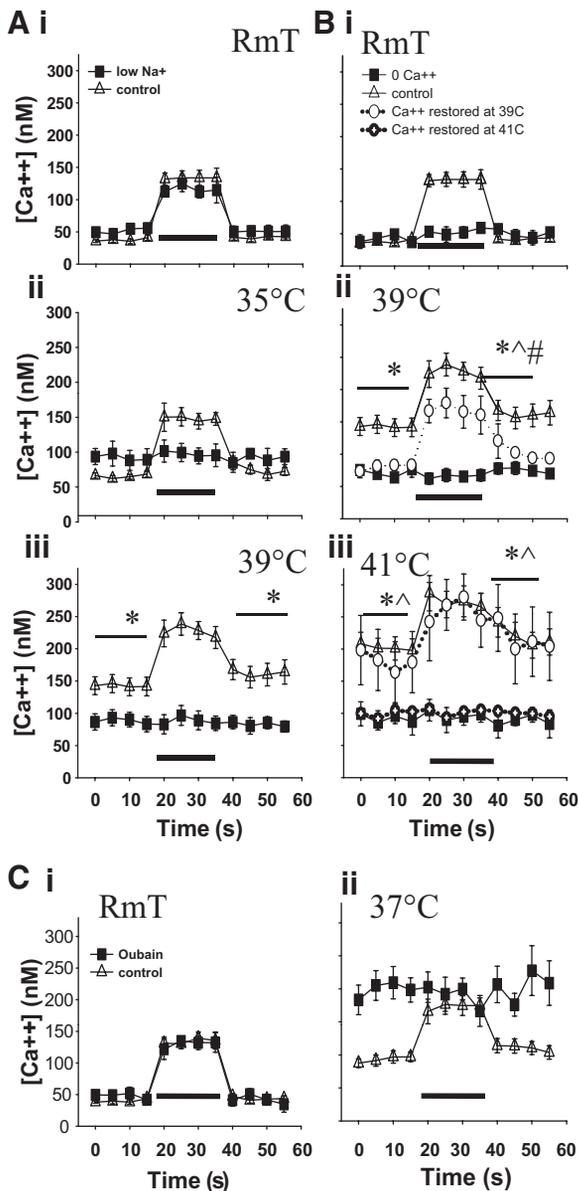
Role of internal calcium stores in maintaining fidelity of evoked calcium response

Figure 6Ai plots the average $[Ca^{2+}]_r$ prior to stimulation at each temperature with × indicating the loss of fidelity in evoked calcium responses in the expanded portion (Fig. 6Aii). This graph reveals that release of calcium from internal stores,

FIG. 5. Disrupting sodium-calcium exchanger or removing extracellular Ca^{2+} prevents normal temperature-induced rise in $[Ca^{2+}]_r$. *A*: extracellular Na^+ was replaced with *N*-methly-D-glucosamine, disrupting the exchange of Na^+ and Ca^{2+}. *i*: at RmT, 0 Na^+ physiological solution did not alter Ca^{2+} regulation. *ii*: at 35°C, the $[Ca^{2+}]_r$ was 92.3 nM ±6.5, significantly higher than controls, and evoked Ca^{2+} responses were disrupted. *iii*: at 39°C, $[Ca^{2+}]_r$ in 0 Na^+ exposed terminals was 88.3 ± 4.9 nM did not respond to stimuli. Significant difference in $[Ca^{2+}]_r$ between control and 0 Na^+ treated terminals (*). *B*: extracellular Ca^{2+} was removed and 1 mM EGTA was added to physiological solution. *i*: lack of extracellular Ca^{2+} did not significantly alter intracellular $[Ca^{2+}]_r$ at RmT but did prevent evoked calcium responses (*n* = 8). *ii*: at 39°C, the $[Ca^{2+}]_r$ in 0 Ca^{2+} preparations was 67.7 ± 3.4 nM (*n* = 4), significantly lower than in controls, which were 142.9 ± 6.9 nM (*n* = 16). In preparations where extracellular Ca^{2+} (0.5 mM) was restored at 39°C (*n* = 4), evoked responses were restored and the $[Ca^{2+}]_r$ following stimulation rose significantly to 97.4 ± 3.1 nM. *Iii*: at 41°C, the $[Ca^{2+}]_r$ in preparations which had Ca^{2+} restored previously, at 39°C was 181.3 ± 22.3 nM (*n* = 4) and was not different from controls, which were 202.0 ± 18.5 nM (*n* = 16), but was significantly higher than preparations maintained in 0 Ca^{2+} solution, which were 91.6 ± 3.3 nM (*n* = 4). When Ca^{2+} was restored at 41°C, Ca^{2+} responses could no longer be evoked, yet $[Ca^{2+}]_r$ was 101.3 ± 7.2 nM (*n* = 4), well below preps exposed to extracellular calcium at lower temperatures. Significant difference between control and 0Ca^{2+} (*), between 0 Ca^{2+} and restored 0 Ca^{2+} (^), and between control and restored Ca^{2+} (#). *C*: ouabain (30 μM) was used to disrupt the Na^+/K^+ exchanger to elevate intracellular Na^+. *i*: at RmT, ouabain had no effect on calcium levels. *ii*: at 37°C, $[Ca^{2+}]_r$ increased from 87.3 ± 4.3 nM (*n* = 16) in controls to 199.1 ± 10.4 nM and stimulation could not evoke a response.
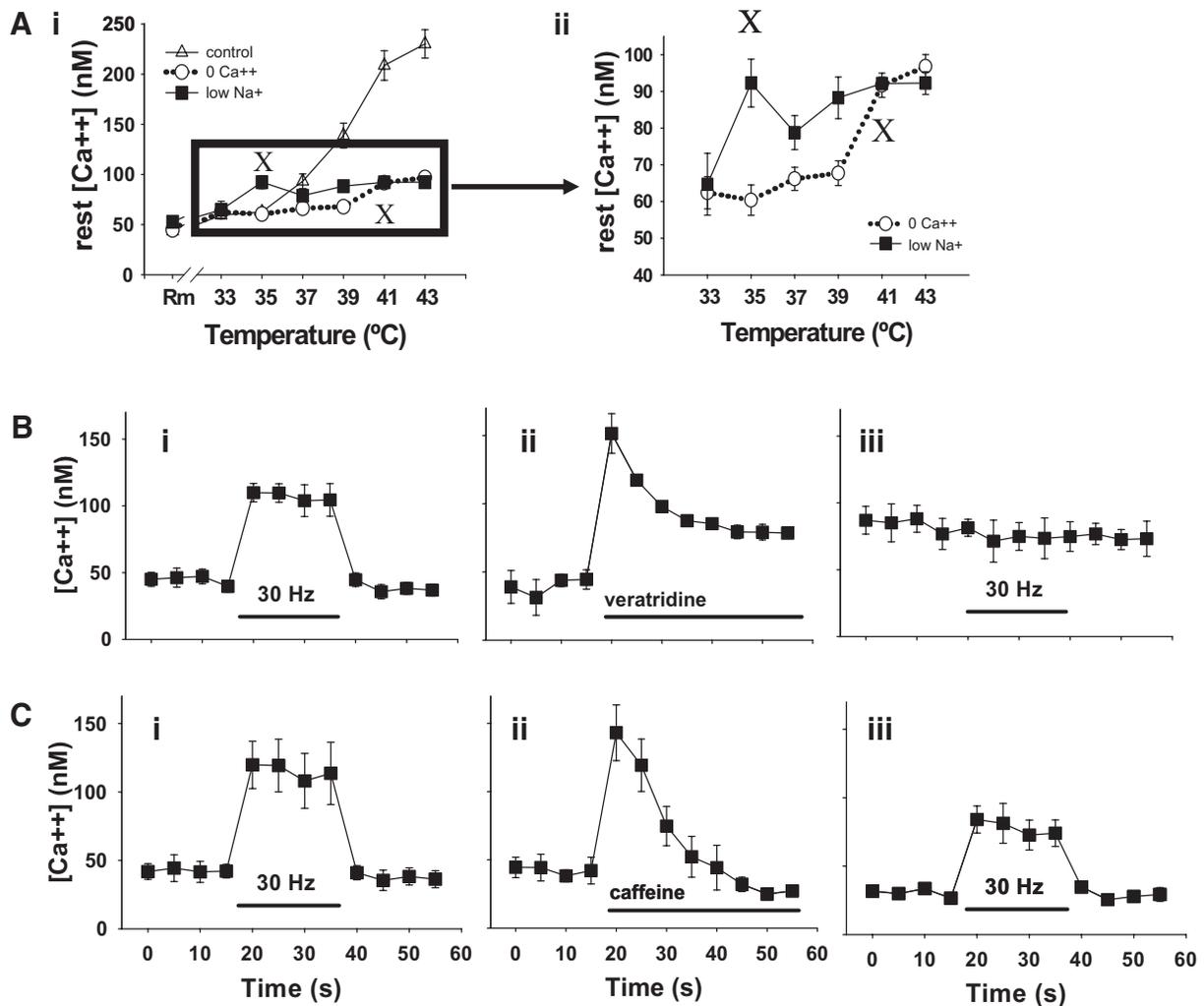
FIG. 6. Release of Ca^{2+} from internal stores coincides with the loss of evoked Ca^{2+} responses. $\times$, the temperatures at which evoked calcium responses are disrupted. *A, i*: effect of increasing temperature on resting calcium concentration in various physiological solutions (■: 0 Na^{+}; ○: 0 Ca^{2+}, △: control). *ii*: a magnification of graph *Ai*. *B*: veratridine induces the release of internal calcium stores, preventing evoked responses. *i*: pre veratridine control. *ii*: veratridine transiently elevated nerve terminal calcium concentration. Intracellular calcium concentration recorded within 5 s of application reveals a peak of 151.5 ± 14.5 nM (*n* = 4), which decayed slowly to 89.6 ± 3.6 nM. *iii*: following 5 min of veratridine washout, calcium concentration remained elevated at 88.2 ± 10.1 nM and calcium responses could not be evoked by stimulation. *C*: caffeine induces release of internal calcium stores without disrupting evoked responses. *i*: precaffeine control calcium response amplitude was 72.7 ± 5.9 nM. *ii*: caffeine transiently elevated nerve terminal calcium concentration. Intracellular calcium concentration recorded within 5 s of application reveals a peak of 142.1 ± 20.4 (*n* = 4), which decays to preapplication levels within 30 s. *iii*: following the application of caffeine, calcium responses can still be evoked with an amplitude of 55.6 ± 7.3 nM, which was significantly smaller than preapplication controls.

seen by a rise to ~90 nM in preparations either lacking extracellular calcium or the means to bring it into the cytosol through the Na^{+}/Ca^{2+} exchanger, correlates with the loss of evoked calcium responses.

To examine the origin of the internal release, endoplasmic reticulum and mitochondrial calcium stores were pharmacologically targeted using caffeine and veratridine. Release of calcium from internal stores can be induced by veratridine in the absence of bath Ca^{2+} (Shakiryanova et al. 2007). The effects of veratridine on calcium-induced vesicle mobility acts independently of the ER, suggesting mitochondrial calcium stores are released (Shakiryanova et al. 2007). To test this directly, we applied veratridine in the absence of bath calcium to *dmiro* mutant terminals, which lack mitochondria in nerve terminals (Guo et al. 2005). The intracellular calcium concentration was 41.8 ± 5.0 nM (*n* = 5) before veratridine application and 41.3 ± 8.5 nM following application. The lack of a

significant difference indicated that there was no release of internal calcium stores. Conversely, application of veratridine, in Ca^{2+}-free solution, to control terminals containing mitochondria resulted in a large transient rise in intracellular calcium to 151.5 ± 14.5 nM (*n* = 4), which decayed slowly to 89.6 ± 3.6 nM within 30 s (Fig. 6*B*). Following the release of calcium from mitochondria, further calcium responses could not be evoked with 30-Hz stimulation once Ca^{2+} was restored to the physiological solution (Fig. b*ig*). Conversely, following release of calcium from the ER with caffeine, calcium levels returned from a peak of 142.1 ± 20.4 (*n* = 4) to peristimulus levels within 20 s. Calcium responses could be successfully evoked following caffeine application (Fig. 6*C*). This suggests that release of calcium specifically from mitochondria precedes the loss of evoked calcium responses.

Release of calcium stores from mitochondria also occurs following the disruption of the respiratory chain using CCCP.

Although glycolysis provides sufficient ATP to maintain resting calcium levels in mutant *dmiro* terminals lacking mitochondrial ATP (Guo et al. 2005), evoked responses were nonetheless disrupted in wild-type larvae by CCCP-induced disruption of mitochondrial function (Fig. 7*A*). Together this suggests that disruption of ATP production following CCCP exposure is not initially responsible for the loss of evoked responses. More likely, the release of internal mitochondrial

A

stores, which would follow CCCP exposure, is responsible for the disruption of evoked calcium responses.

Further support for a role of mitochondrial disruption in hyperthermia-induced block of evoked calcium responses comes from mitochondria-free nerve terminals in *dmiro* mutants (Guo et al. 2005). At room temperature, calcium regulation is relatively normal for brief periods of stimulation, with deficits seen only during prolonged repetitive stimulation (Guo et al. 2005). Similar deficits were seen when we exposed *dmiro* terminals to very mild increases in temperature. At 33°C, $[Ca^{+2}]_r$ was significantly elevated above controls (P[ry+,FRT] 83B,ry−) revealing a profound reduction in the ability to maintain calcium homeostasis (Fig. 7*Bi*). Interestingly, Ca^{+2} responses could still be evoked even when $[Ca^{+2}]_r$ was significantly higher than 200 nM for several minutes (Fig. 7*B, ii–iii*), which in all other terminals was a level that coincided with the loss of evoked Ca^{+2} responses.
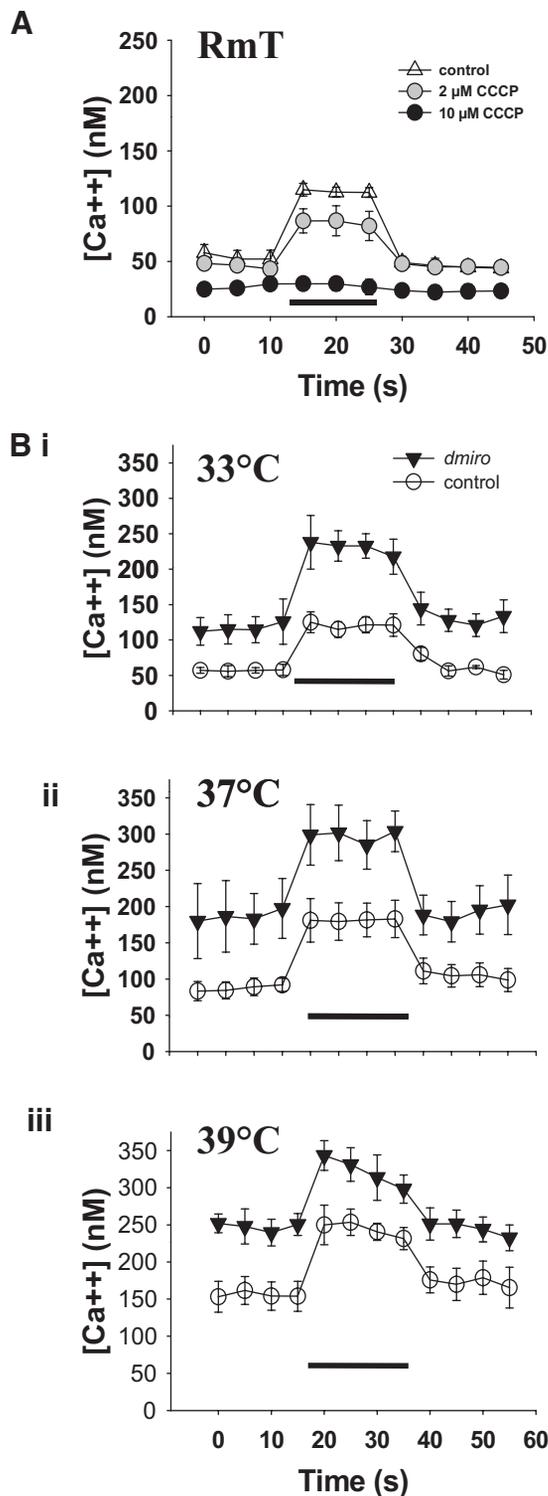
DISCUSSION

We examined roles in synaptic thermotolerance of mechanisms responsible for maintaining Ca^{2+} homeostasis. ATP limits nerve terminal Ca^{2+} regulation during hyperthermia. Disrupting ATP production either genetically or pharmacologically reduces the nerve terminal's ability to maintain synaptic function during hyperthermic stress, whereas fortifying energy levels with exogenous ATP enhances it. Experiments using nerve terminals lacking mitochondria suggest that glycolysis produces sufficient ATP to maintain competent synaptic function under nonstressful conditions (Guo et al. 2005; Verstreken et al. 2005). We show that during hyperthermia, both sources of energy production are required for maintaining presynaptic calcium homeostasis and for maintaining the fidelity of evoked Ca^{2+} responses and evoked synaptic transmission.

Our evidence indicates that during hyperthermia, the Na^+/Ca^{2+} exchanger, operating in the reverse mode, brings calcium into the cytosol. This imposes an energetic demand on the nerve terminal to clear the Ca^{2+}. After a prolonged exposure, Ca^{2+} stores are released and evoked synaptic responses are lost. Releasing Ca^{2+} specifically from mitochondria, but not from the ER, disrupts synaptic responses.

Role of ATP-dependent calcium clearance in synaptic thermotolerance

At 43°C, nerve stimulation failed to evoke either a Ca^{2+} response or an excitatory postsynaptic potential in control preparations. At this temperature, the terminal is exposed to a prolonged elevation of $[Ca^{2+}]_r$ above 200 nM with no respite

FIG. 7. Effect of mitochondrial disruption on nerve terminal calcium regulation. *A*: CCCP disrupted evoked calcium responses without a maintained elevation in $[Ca^{+2}]_r$. Following the application of 2 μM CCCP, the amplitude of evoked calcium responses decreased from 95.0 ± 5.6 to 39.18 ± 7.2 nM. Following the application of 10 μM CCCP, $[Ca^{+2}]_r$ was 26.8 ± 2.9 nM, and calcium responses could not be evoked (*n* = 6). *B*: calcium regulation in *Drosophila dmiro* neuromuscular nerve terminals. *i*: at 33°C, $[Ca^{+2}]_r$ in *dmiro* mutant terminals was 116.9 ± 10.9 nM, well above controls at 56.9 ± 2.9 nM. *ii*: at 37°C, $[Ca^{+2}]_r$ in *dmiro* mutant terminals was 189.0 ± 20.5 nM, significantly higher than controls at 89.3 ± 12.1 nM. *iii*: at 39°C, $[Ca^{+2}]_r$ in *dmiro* terminals (*n* = 6) is elevated to 247.5 ± 8.0 nM, well above control levels, which was 155.7 ± 9.5 nM (P[ry+,FRT] 83B,ry−; *n* = 6). At both 37 and 39°C, $[Ca^{+2}]_r$ is above 200 nM, yet calcium responses are maintained.

(Klose et al. 2008). Disruption of either energy-dependent calcium clearing mechanism, the SERCA or the PMCA, accelerated the temperature-induced elevation in $[Ca^{2+}]_r$ and reduced the upper temperature limit of both evoked Ca^{2+} responses and synaptic transmission. Furthermore, disrupting glycolysis with iodoacetate, an inhibitor of glyceraldehyde-3-phosphate dehydrogenase (Kockskämper et al. 2005), mimicked the disruptive effects of blocking the ATP-dependent calcium pumps, whereas supplementing energy stores with exogenous ATP protected synaptic function at elevated temperatures. Together these observations reveal that ATP-dependent calcium regulating mechanisms limit synaptic function at high temperatures.

Mammalian N-type calcium channels are up-regulated by phosphorylation (Martin et al. 2006). Thus ATP levels might regulate N-type calcium channel function in *Drosophila* nerve terminals. ATP depletion has been shown to activate *Drosophila* TRP channels that could also disrupt neuronal function (Agam et al. 2004). Targeted motoneuronal expression of hsp70 has been shown to thermoprotect calcium regulation in this preparation (Klose et al. 2008). In mammalian fast twitch muscle, temperature-induced decreases in SERCA activity can be attenuated by exogenous hsp70, thereby protecting calcium regulation (Tupling et al. 2004). The available evidence suggests that hyperthermia affects presynaptic ATP levels and/or ATP utilization, reducing the nerve terminal's ability to maintain low $[Ca^{2+}]_r$, evoked calcium responses, and synaptic transmission.

Na^+/Ca^{2+} exchanger and extracellular Ca^{2+} elevate $[Ca^{2+}]_r$ during hyperthermia

As temperature increases, Ca^{2+} accumulates in the nerve terminal cytosol,(Klose et al. 2008). Lack of extracellular Ca^{2+} prevents the typical rise in $[Ca^{2+}]_r$ seen at high temperatures; this rise also requires normal extracellular Na^+ levels, suggesting involvement of the Na^+/Ca^{2+} exchanger. Normally the Na^+/Ca^{2+} exchanger extrudes one Ca^{2+} ion from the cell by exchanging it for three Na^+ ions (DiPolo and Beauge 2006). However, physiologically stressful conditions can induce a reversed mode of function (Zhong et al. 2001). In human epidermal cells exposed to high temperatures, Ca^{2+} enters and Na^+ is cleared by the Na^+/Ca^{2+} exchanger revealing a hyperthermia-reversed mode of function (Kiang et al. 1992). Reverse mode function of the exchanger can also be induced by oxidative stress (Yang et al. 2004). This reversal is believed to be regulated by the Na^+ gradient (Watanabe et al. 2006). Whether this is protective or disruptive is controversial. Calcium imaging experiments using BHK cells transfected with Na^+/Ca^{2+} exchanger isoform 3 suggests that the reversal is protective against hypoxia-induced cell death (Secondo et al. 2007), whereas in another study, blocking the reverse mode of the exchanger was protective against ischemia-reperfusion pathologies (Elias et al. 2001). The reversal of function may be initially protective, acting to slow the buildup of Na^+. Abnormally high Na^+ increases osmotic pressure and can elevate reactive oxygen species, induce cytoskeletal rearrangement, inhibit transcription and translation, damage both DNA and proteins, and disrupt mitochondrial function (Takeo and Tanonaka 2004). Elevated Ca^{2+} activates calpain inducing the activation of caspases (Chan and Mattson 1999) and, like Na^+,

it can cause permeability transition in mitochondria (Dong et al. 2005; Newmeyer and Ferguson-Miller 2003). Our data support the hypothesis that the reverse mode of the Na^+/Ca^{2+} exchanger promotes entry of extracellular Ca^{2+} into the nerve terminals at high temperatures.

Hyperthermia-induced disruption of evoked Ca^{2+} response—role of Na^+ and Ca^{2+}

Elevation of $[Ca^{2+}]_r$ above 200 nM, either directly with a calcium ionophore or indirectly with hyperthermia, disrupted evoked Ca^{2+} responses. High cytosolic Ca^{2+} can negatively modulate Ca^{2+}-channel function (Burgoyne and Weiss 2001; Liang et al. 2003; Zuhlke et al. 1999). However, responses could be disrupted without a large prolonged rise in $[Ca^{2+}]_r$ as observed in experiments in which the Na^+/Ca^{2+} exchanger was disrupted. In these experiments, loss of evoked calcium responses occurred with only a small elevation in $[Ca^{2+}]_r$ to ~90 nM, which is normally permissive to evoked calcium responses. This reveals that prolonged elevation of $[Ca^{2+}]_r$ above 200 nM is not required to disrupt evoked calcium responses if $[Na^+]_r$ is abnormally high and suggests that disrupting of Na^+ clearance may contribute significantly to impairment of nerve terminal physiology.

Ouabain, which disrupts the Na^+/K^+-ATPase thereby reducing the clearance of intracellular Na^+ (Rahamimoff et al. 1980), facilitated the temperature-induced rise in $[Ca^{2+}]_r$ and loss of evoked responses. This suggests that the nerve terminal reacts to an increased Na^+ load by elevating Ca^{2+} and supports the idea that excessively elevated Na^+ can disrupt Ca^{2+} regulation. Elevated Na^+ depolarizes the axon and could render the nerve terminal nonexcitable to further stimulation if the terminals space constant were altered sufficiently. However, this is not likely because hyperthermic terminals lacking mitochondria remained excitable even after ionic homeostasis and synaptic transmission had been disrupted. Veratridine, which elevates intracellular Na^+ through persistent activation of Na^+ channels (Suzuki and Wu 1984), also caused an elevation in intracellular calcium concentration but only in terminals containing mitochondria. Regulatory roles for Na^+ in synaptic function were supported by earlier studies of synaptic modulation (Atwood et al. 1975; Birks and Cohen 1968; Sherman and Atwood 1971). Na^+ can initiate the release of Ca^{2+} from mitochondria in both vertebrate and invertebrate neuromuscular junctions (Rahamimoff et al. 1980; Zhong et al. 2001). This effect has been used to explain the potentiation of transmitter release after vigorous stimulation and in fact may underlie the so-called "augmentation" phase of enhanced release (Rahamimoff et al. 1980; Zhong et al. 2001). During hyperthermia, Na^+ entry may surpass the normal physiological limit and go beyond the potentiation of release, leading instead to disruptive Ca^{+2} levels and failure of synaptic transmission.

In all manipulations, EJP amplitude decreased with increasing temperature, and prior to transmission failure, a large increase in spontaneous transmitter release could be observed. In all manipulations except for those involving mutant *dmiro* terminals lacking mitochondria, loss of evoked Ca^{2+} responses coincided with the loss of synaptic transmission. Exposure of *dmiro* terminals to several minutes of $[Ca^{2+}]_r$ above 200 nM disrupted synaptic transmission but not evoked calcium responses. Thus elevated $[Ca^{2+}]_r$ is not sufficient by itself to disrupt evoked calcium

responses in all cases; the presence of mitochondria in the nerve terminal is also required for the loss of the evoked response. Thus we suggest hyperthermia-induced elevations in Na^+ promote the release of Ca^{2+} from mitochondrial stores and ultimately the disruption of evoked Ca^{2+} responses.

Typically, the temperature-induced disruption of EJPs is preceded by an increase in spontaneous release of transmitter and occurs along with the loss of evoked Ca^{2+} responses regardless of treatment (Klose et al. 2008). This suggests synaptic function is lost due to disruption of presynaptic Ca^{2+} handling. However, in experiments using *dmiro* mutants, synaptic transmission fails before the loss of evoked calcium responses, suggesting that other stress-sensitive synaptic mechanisms can determine the thermotolerance level of synaptic transmission. Both vesicle exocytosis and endocytosis are phosphorylation-dependent processes (Robinson et al. 1993; Wang et al. 2004) that could limit the thermotolerance of synaptic function in the absence of mitochondria. In wild-type nerve terminals, the temperature-induced loss of evoked Ca^{2+} responses requires the presence of mitochondria, further suggesting that mitochondrial mechanisms sensitive to the disruption of ionic homeostasis are responsible for the disruption of the Ca^{2+} responses and synaptic transmission. In several other organisms, spontaneous release of transmitter can be induced by alterations in cytosolic Ca^{2+} caused by either pharmacological disruption of mitochondria or disruption of the sodium-calcium exchanger (Alnaes and Rahamimoff 1975; Scotti et al. 1999).

The small temperature-induced rise in Ca^{2+} to ~90 nM that occurred in the absence of extracellular Ca^{2+} revealed a stress-induced release from internal stores, likely the result of elevated intracellular Na^+. This release of Ca^{2+} correlated with the loss of evoked Ca^{2+} responses. Release of Ca^{2+} from mitochondria with either veratridine (Shakiryanova et al. 2007) or with the mitochondrial transport chain uncoupler CCCP (Nishikawa et al. 2000) prevented further evoked calcium responses without prolonged rises in $[Ca^{2+}]_r$ above 200 nM; release of calcium from the endoplasmic reticulum with caffeine did not prevent further evoked calcium responses. Disruption of mitochondria interrupts oxidative phosphorylation and releases reactive oxygen species, degradative enzymes, and several other factors in addition to calcium (Dong et al. 2005). Because evoked calcium responses occur in terminals lacking mitochondria (Guo et al. 2005; Verstreken et al. 2005), loss of oxidative phosphorylation is likely not directly responsible for the loss of evoked responses. Thus disruption of mitochondrial function and the subsequent release of internal contents into nerve terminal cytosol are sufficient and likely necessary for the temperature-induced loss of evoked calcium response.

In summary, as temperature increases, the Na^+/Ca^{2+} exchanger reverses function bringing extracellular Ca^{2+} into the nerve terminal to reduce the Na^+ load. The metabolic burden of maintaining ionic homeostasis at high temperatures leaves the ATP-dependent pumps unable to maintain synaptic function. Thus insufficient ATP likely causes the loss of ionic homeostasis, which disrupts mitochondrial function causing the release of its internal stores, which in turn induces spontaneous release of neurotransmitter and leads to the loss of evoked calcium responses and the failure of synaptic transmission.

REFERENCES

Agam K, Frechter S, Minke B. Activation of the Drosophila TRP and TRPL channels requires both Ca^{2+} and protein dephosphorylation. *Cell Calcium* 35: 87–105, 2004.

Alnaes E, Rahamimoff R. On the role of mitochondria in transmitter release from motor nerve terminals. *J Physiol* 248: 285–306, 1975.

Altier C, Khosravani H, Evans RM, Hameed S, Peloquin JB, Vartian BA, Chen L, Beedle AM, Ferguson SS, Mezghrani A, Dubel SJ, Bourinet E, McRory JE, Zamponi GW. ORL1 receptor-mediated internalization of N-type calcium channels. *Nat. Neurosci* 9: 31–40, 2005.

Atwood HL, Swenarchuk LE, Gruenwald CR. Long-term synaptic facilitation during sodium accumulation in nerve terminals. *Brain Res* 100: 198–202, 1975.

Babsky A, Hekmatyar SK, Gorski T, Nelson DS, Bansal N. Heat-induced changes in intracellular Na^+, pH and bioenergetic status in superfused RIF-1 tumor cells determined by 23Na and 31P magnetic resonance spectroscopy. *Int J Hyperthermia* 21: 141–158, 2005.

Bao H, Daniels RW, MacLeod GT, Charlton MP, Atwood HL, Zhang B. AP180 maintains the distribution of synaptic and vesicle proteins in the nerve terminal and indirectly regulates the efficacy of Ca^{2+}-triggered exocytosis. *J Neurophysiol* 94: 1888–1903, 2005.

Bickler PE, Buck LT. Adaptations of vertebrate neurons to hypoxia and anoxia: maintaining critical Ca^{2+} concentrations. *J Exp Biol* 201: 1141–1152, 1998.

Birks RI, Cohen MW. The action of sodium pump inhibitors on neuromuscular transmission. *Proc R Soc Lond B Biol Sci* 170: 381–399, 1968.

Brand AH, Perrimon N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 18: 401–415, 1993.

Bronk P, Nie Z, Klose MK, Dawson-Scully K, Zhang J, Robertson RM, Atwood HL, Zinsmaier KE. The multiple functions of cysteine-string protein analyzed at *Drosophila* nerve terminals. *J Neurosci* 25: 2204–2214, 2005.

Bronk P, Wenniger JJ, Dawson-Scully K, Guo X, Hong S, Atwood HL, Zinsmaier KE. *Drosophila* Hsc70-4 is critical for neurotransmitter exocytosis in vivo. *Neuron* 30: 475–488, 2001.

Buck LT, Pamenter ME. Adaptive responses of vertebrate neurons to anoxia—matching supply to demand. *Respir Physiol Neurobiol* 154: 226–240, 2006.

Budd SL. Mechanisms of neuronal damage in brain hypoxia/ischemia: focus on the role of mitochondrial calcium accumulation. *Pharmacol Ther* 80: 203–229, 1998.

Burg MB, Ferraris JD, Dmitrieva NI. Cellular response to hyperosmotic stresses. *Physiol Rev* 87: 1441–1474, 2007.

Burgoyne RD, Weiss JL. The neuronal calcium sensor family of Ca^{2+}-binding proteins. *Biochem J* 353: 1–12, 2001.

Chan SL, Mattson MP. Caspase and calpain substrates: roles in synaptic plasticity and cell death. *J Neurosci Res* 5: 167–190, 1999.

Cohen O, Kanana H, Zoizner R, Gross C, Meiri U, Stern MD, Gerstenblith G, Horowitz M. Altered Ca^{2+} handling and myofilament desensitization underlie cardiomyocyte performance in normothermic and hyperthermic heat-acclimated rat hearts. *J Appl Physiol* 103: 266–275, 2007.

De Koninck P, Schulman H. Sensitivity of CaM kinase II to the frequency of Ca^{2+} oscillations. *Science* 279: 227–230, 1998.

DiPolo R, Beaugé L. Sodium/calcium exchanger: influence of metabolic regulation on ion carrier interactions. *Physiol Rev* 86: 155–203, 2006.

Dong Z, Saikumar P, Weinberg JM, Venkatachalam MA. Calcium in cell injury and death. *Annu Rev Pathol* 1: 405–434, 2005.

Elias CL, Lukas A, Shurraw S, Scott J, Omelchenko A, Gross GJ, Hnatowich M, Hryshko LV. Inhibition of Na^+/Ca^{2+} exchange by KB-

R7943: transport mode selectivity and antiarrhythmic consequences. *Am J Physiol Heart Circ Physiol* 281: H1334–H1345, 2001.

Gerner EW. Thermal dose and time-temperature factors for biological responses to heat shock. *Int J Hyperthermia* 3: 319–327, 1987.

Gong WJ, Golic KG. Loss of Hsp70 in *Drosophila* is pleiotropic, with effects on thermotolerance, recovery from heat shock and neurodegeneration. *Genetics* 172: 275–286, 2006.

Gonzalez-Mendez RR, Hahn GM. Effects of hyperthermia on the intracellular pH and membrane potential of Chinese hamster ovary cells. *Int J Hyperthermia* 5: 69–84, 1989.

Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 260: 3440–3450, 1985.

Guo X, Macleod GT, Wellington A, Hu F, Panchumarthi S, Schoenfield M, Marin L, Charlton MP, Atwood HL, Zinsmaier KE. The GTPase dMiro is required for axonal transport of mitochondria to *Drosophila* synapses. *Neuron* 47: 379–393, 2005.

Hay JC, Scheller RH. SNAREs and NSF in targeted membrane fusion. *Curr Opin Cell Biol* 9: 505–512, 1997.

Hsu SF, Augustine GJ, Jackson MB. Adaptation of Ca^{2+}-triggered release in synaptic terminals. *Neuron* 17: 501–512, 1996.

Kockskämper J, Zima AV, Blatter LA. Modulation of sarcoplasmic reticulum Ca^{2+} release by glycolysis in cat atrial myocytes. *J Physiol* 564: 697–714, 2005.

Karunanithi S, Barclay JW, Brown IR, Robertson RM, Atwood HL. Enhancement of presynaptic performance in transgenic *Drosophila* overexpressing heat shock protein Hsp70. *Synapse* 44: 8–14, 2002.

Karunanithi S, Barclay JW, Robertson RM, Brown IR, Atwood HL. Neuroprotection at *Drosophila* synapses conferred by prior heat shock. *J Neurosci* 19: 4360–4369, 1999.

Katschinski DM, Boos K, Schindler SG, Fandrey J. Pivotal role of reactive oxygen species as intracellular mediators of hyperthermia-induced apoptosis. *J Biol Chem* 275: 21094–21098, 2000.

Kiang JG, Koenig ML, Smallridge RC. Heat shock increases cytosolic free Ca^{2+} concentration via $Na(^+)$-Ca^{2+} exchange in human epidermoid A 431 cells. *Am J Physiol Cell Physiol* 263: C30–C38, 1992.

Klose MK, Armstrong G, Robertson RM. A role for the cytoskeleton in heat-shock-mediated thermoprotection of locust neuromuscular junctions. *J Neurobiol* 60: 453–462, 2004.

Klose MK, Atwood HL, Robertson RM. Hyperthermic preconditioning of presynaptic calcium regulation in *Drosophila*. *J Neurophysiol* 99: 2420–2430, 2008.

Klose MK, Chu D, Xiao C, Seroude L, Robertson RM. Heat shock-mediated thermoprotection of larval locomotion compromised by ubiquitous overexpression of Hsp70 in *Drosophila melanogaster*. *J Neurophysiol* 94: 3563–3572, 2005.

Kuromi H, Kidokoro Y. Selective replenishment of two vesicle pools depends on the source of Ca^{2+} at the *Drosophila* synapse. *Neuron* 35: 333–343, 2002.

Lnenicka GA, Grizzaffi J, Lee B. Rumpal N Ca^{2+} dynamics along identified synaptic terminals in *Drosophila* larvae. *J Neurosci* 26: 12283–12293, 2006.

Liang H, DeMaria CD, Erickson MG, Mori MX, Alseikhan BA, Yue DT. Unified mechanisms of Ca^{2+} regulation across the Ca^{2+} channel family. *Neuron* 39: 951–960, 2003.

Macleod GT, Chen L, Karunanithi S, Peloquin JB, Atwood HL, McRory JE, Zamponi GW, Charlton MP. The Drosophila cac^ts2 mutation reduces presynaptic Ca^{2+} entry and defines an important element in $Ca_v2.1$ channel inactivation. *Eur J Neurosci* 23: 3230–3244, 2006.

Macleod GT, Hegström-Wojtowicz M, Charlton MP, Atwood HL. Fast calcium signals in *Drosophila* motor neuron terminals. *J Neurophysiol* 88: 2659–2663, 2002.

Macleod GT, Suster ML, Charlton MP, Atwood HL. Single neuron activity in the *Drosophila* larval CNS detected with calcium indicators. *J Neurosci Methods* 127: 167–178, 2003.

Martin SW, Butcher AJ, Berrow NS, Richards MW, Paddon RE, Turner DJ, Dolphin AC, Sihra TS, Fitzgerald EM. Phosphorylation sites on calcium channel alpha1 and beta subunits regulate ERK-dependent modulation of neuronal N-type calcium channels. *Cell Calcium* 39: 275–292, 2006.

Millar AG, Zucker RS, Ellis-Davies GC, Charlton MP, Atwood HL. Calcium sensitivity of neurotransmitter release differs at phasic and tonic synapses. *J Neurosci* 25: 3113–3125, 2005.

Mozhayeva MG, Matos MF, Liu X, Kavalali ET. Minimum essential factors required for vesicle mobilization at hippocampal synapses. *J Neurosci* 24: 1680–1688, 2004.

Newmeyer DD, Ferguson-Miller S. Mitochondria: releasing power for life and unleashing the machineries of death. *Cell* 112: 481–490, 2003.

Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Giardino I, Brownlee M. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* 404: 787–790, 2000.

Orrenius S, McConkey DJ, Bellomo G, Nicotera P. Role of Ca^{2+} in toxic cell killing. *Trends Pharmacol Sci* 10: 281–285, 1989.

Pant HC, Gallant PE, Gould R, Gainer H. Distribution of calcium-activated protease activity and endogenous substrates in the squid nervous system. *J Neurosci* 2: 1578–1587, 1982.

Parkes TL, Elia AJ, Dickinson D, Hilliker AJ, Phillips JP, Boulianne GL. Extension of *Drosophila* lifespan by overexpression of human SOD1 in motorneurons. *Nat Genet* 19:171–174, 1998.

Parsell DA, Taulien J, Lindquist S. The role of heat-shock proteins in thermotolerance. *Philos Trans R Soc Lond B Biol Sci* 339: 279–285, 1993.

Ravin R, Spira ME, Parnas H, Parnas I. Simultaneous measurement of intracellular Ca^{2+} and asynchronous transmitter release from the same crayfish bouton. *J Physiol* 501: 251–262, 1997.

Rahamimoff R, Lev-Tov A, Meiri H. Primary and secondary regulation of quantal transmitter release: calcium and sodium. *J Exp Biol* 89: 5–18, 1980.

Reiff DF, Ihring A, Guerrero G, Isacoff EY, Joesch M, Nakai J, Borst A. In vivo performance of genetically encoded indicators of neural activity in flies. *J Neurosci* 25: 4766–4778, 2005.

Ritossa F. A new puffing pattern induced by temperature shock and DNP in *Drosophila*. *Experientia* 18: 571–573, 1962.

Robinson PJ, Sontag JM, Liu JP, Fykse EM, Slaughter C, McMahon H, Südhof TC, Dynamin GT. Pase regulated by protein kinase C phosphorylation in nerve terminals. *Nature* 365: 163–166, 1993.

Scorrano L, Ashiya M, Buttle K, Weiler S, Oakes SA, Mannella CA, Korsmeyer SJ. A distinct pathway remodels mitochondrial cristae and mobilizes cytochrome c during apoptosis. *Dev Cell* 2: 55–67, 2002.

Scotti AL, Chatton JY, Reuter H. Roles of $Na(^+)$-Ca^{2+} exchange and of mitochondria in the regulation of presynaptic Ca^{2+} and spontaneous glutamate release. *Philos Trans R Soc Lond B Biol Sci* 354: 357–364, 1999.

Secondo A, Staiano IR, Scorziello A, Sirabella R, Boscia F, Adornetto A, Canzoniero LM, Di Renzo G, Annunziato L. The Na^+/Ca^{2+} exchanger isoform 3 (NCX3) but not isoform 2 (NCX2) and 1 (NCX1) singly transfected in BHK cells plays a protective role in a model of in vitro hypoxia. *Ann NY Acad Sci* 1099: 481–485, 2007.

Shakiryanova D, Klose MK, Zhou Y, Gu T, Deitcher DL, Atwood HL, Hewes RS, Levitan ES. Presynaptic ryanodine receptor-activated calmodulin kinase II increases vesicle mobility and potentiates neuropeptide release. *J Neurosci* 27: 7799–7806, 2007.

Sherman RG, Atwood HL. Synaptic facilitation: long-term neuromuscular facilitation in crustaceans. *Science* 171: 1248–1250, 1971.

Solomon JM, Rossi JM, Golic K, McGarry T, Lindquist S. Changes in hsp70 alter thermotolerance and heat-shock regulation in *Drosophila. New Biol* 3: 1106–1120, 1991.

Stefani G, Onofri F, Valtorta F, Vaccaro P, Greengard P, Benfenati F. Kinetic analysis of the phosphorylation-dependent interactions of synapsin I with rat brain synaptic vesicles. *J Physiol* 504: 501–515, 1997.

Stewart BA, Mohtashami M, Rivlin P, Deitcher DL, Trimble WS, Boulianne GL. Dominant-negative NSF2 disrupts the structure and function of *Drosophila* neuromuscular synapses. *J Neurobiol* 51: 261–271, 2002.

Stys PK. White matter injury mechanisms. *Curr Mol Med* 4: 113–130, 2004.

Suzuki N, Wu CF. Altered sensitivity to sodium channel-specific neurotoxins in cultured neurons from temperature-sensitive paralytic mutants of *Drosophila*. *J Neurogenet* 1: 225–238, 1984.

Szigeti G, Banyasz T, Magyar J, Kortvely A, Szigligeti P, Kovacs L, Jednakovits A, Nanasi P. Effects of bimoclomol, the novel heat shock protein coinducer, in dog ventricular myocardium. *Life Sci* 67: 73–79, 2000.

Takeo S, Tanonaka K. Na^+ overload-induced mitochondrial damage in the ischemic heart. *Can J Physiol Pharmacol* 82: 1033–1043, 2004.

Tank DW, Regehr WG, Delaney KR. A quantitative analysis of presynaptic calcium dynamics that contribute to short-term enhancement. *J Neurosci* 15: 7940–7952, 1995.

Torrie LS, Radford JC, Southall TD, Kean L, Dinsmore AJ, Davies SA, Dow JA. Resolution of the insect ouabain paradox. *Proc Natl Acad Sci USA* 10: 13689–13693, 2004.

Tupling AR, Gramolini AO, Duhamel TA, Kondo H, Asahi M, Tsuchiya SC, Borrelli MJ, Lepock JR, Otsu K, Hori M, MacLennan DH, Green HJ. HSP70 binds to the fast-twitch skeletal muscle sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA1a) and prevents thermal inactivation. *J Biol Chem* 279: 52382–52389, 2004.

Verstreken P, Ly CV, Venken KJ, Koh TW, Zhou Y, Bellen HJ. Synaptic mitochondria are critical for mobilization of reserve pool vesicles at *Drosophila* neuromuscular junctions. *Neuron* 47: 365–378, 2005.

Vigh L, Literati PN, Horvath I, Torok Z, Balogh G, Glatz A, Kovacs E, Boros I, Ferdinandy P, Farkas B, Jaszlits L, Jednakovits A, Koranyi L, Maresca B. Bimoclomol: a nontoxic, hydroxylamine derivative with stress protein-inducing activity and cytoprotective effects. *Nat Med* 3: 1150–1154, 1997.

Wang JW, Wong AM, Flores J, Vosshall LB, Axel R. Two-photon calcium imaging reveals an odor-evoked map of activity in the fly brain. *Cell* 112: 271–282, 2003.

Wang P, Saraswati S, Guan Z, Watkins CJ, Wurtman RJ, Littleton JT. A *Drosophila* temperature-sensitive seizure mutant in phosphoglycerate kinase disrupts ATP generation and alters synaptic function. *J Neurosci* 24: 4518–4529, 2004.

Watanabe Y, Koide Y, Kimura J. Topics on the Na^+/Ca^{2+} exchanger: pharmacological characterization of Na^+/Ca^{2+} exchanger inhibitors. *J Pharmacol Sci* 102: 7–16, 2006.

Wong K, Karunanithi S, Atwood HL. Quantal unit populations at the *Drosophila* larval neuromuscular junction. *J Neurophysiol* 82: 1497–1511, 1999.

Yang KT, Pan SF, Chien CL, Hsu SM, Tseng YZ, Wang SM, Wu ML. Mitochondrial Na^+ overload is caused by oxidative stress and leads to activation of the caspase 3-dependent apoptotic machinery. *FASEB J* 18: 1442–1444, 2004.

Zhong N, Beaumont V, Zucker RS. Roles for mitochondrial and reverse mode Na^+/Ca^{2+} exchange and the plasmalemma Ca^{2+} ATPase in post-tetanic potentiation at crayfish neuromuscular junctions. *J Neurosci* 21: 9598–607, 2001.

Zucker RS, Regehr WG. Short-term synaptic plasticity. *Annu Rev Physiol* 64: 355–405, 2002.

Zuhlke RD, Pitt GS, Deisseroth K, Tsien RW, Reuter H. Calmodulin supports both inactivation and facilitation of L-type calcium channels. *Nature* 399: 159–162, 1999.