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Hyperthermic Preconditioning of Presynaptic Calcium Regulation in *Drosophila*

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Klose MK, Atwood HL, Robertson RM. Hyperthermic preconditioning of presynaptic calcium regulation in *Drosophila*. *J Neurophysiol* 99: 2420–2430, 2008. First published February 13, 2008; doi:10.1152/jn.01251.2007. We examined the thermosensitivity of calcium regulation in *Drosophila* larval neuromuscular junctions, testing effects of prior heat shock and Hsp70 expression. Motor neurons were loaded with either the ratiometric indicator Fura-dextran or the nonratiometric indicator Oregon Green bis-(*o*-aminophenoxy)-*N,N,N′,N′*-tetraacetic acid to monitor parameters of calcium regulation as temperature increased. Nerve terminals treated to a prior heat shock, and those of transgenic flies expressing higher than normal levels of Hsp70, were better able to maintain near-normal resting calcium concentrations, calcium influx, and calcium clearance at higher temperatures. Synaptic transmission was also protected by prior heat shock and by higher than normal Hsp70 expression. Thus the thermal limit of synaptic transmission may be directly linked to the stability of calcium regulation.

INTRODUCTION

During hyperthermic insults in a variety of cell types, resting calcium concentration increases (Kiang et al. 1992), pH decreases (Babsky et al. 2005), reactive oxygen species are generated (Katschinski et al. 2000), membrane potential is altered (Gonzalez-Mendez and Hahn 1989), and most protein synthesis is downregulated, while several heat shock proteins (Hsps) are upregulated (Neal et al. 2006; Parsell et al. 1993; Solomon et al. 1991). The last feature is dramatically evident in *Drosophila*, where the heat shock response was first discovered (Ritossa 1962). *Drosophila* locomotor behavior, motor pattern generation, and synaptic transmission are disrupted by hyperthermic stress; the thermal sensitivity of each of these functions is plastic (Karunanithi et al. 1999; Klose et al. 2005). Whether hyperthermia-induced failure of synaptic function is due to disruption of critical protein function, lack of energy, accumulation of reactive oxygen species, disruption in Ca^{2+} regulation, or interactions involving of several of these factors is unclear.

A prior moderate thermal stress or heat shock (HS) can induce mechanisms that protect cell viability and function against subsequent more extreme stressors (Karunanithi et al. 1999; Parsell et al. 1993; Solomon et al. 1991). Pharmacological induction of heat shock protein (Hsp) synthesis has revealed cytoprotective benefits against several stressors (Brown 2007; Szigeti et al. 2000; Vigh et al. 1997). Current evidence supports the involvement of Hsps, particularly Hsp70 in stress protection of synaptic transmission at high temperatures. Both gain-of-function (Karunanithi et al. 2002; Xiao et al. 2007) and loss-of-function studies (Bronk et al. 2001; Gong and Golic 2006) suggest a role for this chaperone in thermotolerance of synaptic transmission. The mechanisms responsible for this protection are only beginning to be unraveled, although a role for presynaptic calcium dynamics has been suggested (Barclay and Robertson 2003).

A recurring theme among stress-induced pathologies is the loss of normal cytosolic calcium regulation (Bickler and Buck 1998; Buck and Parmenter 2006; Stys 2004). Pathological elevations in free cytosolic calcium can activate catabolic enzymes, which subsequently disrupt the integrity of the cytoskeleton, fragment DNA, damage organelles such as the nucleus and mitochondria, and lead to cell death (Budd 1998; Orrenius et al. 1989). Thus preventing large increases in resting intracellular calcium ion concentration ($[Ca^{2+}]_i$) enhances cell survival.

We hypothesized that the HS response protects mechanisms of Ca^{2+} regulation at high temperatures through actions of Hsp70, which improves the thermotolerance of synaptic transmission. In the present study, we tested this hypothesis by examining the thermosensitivity of calcium homeostasis and testing for HS-mediated and Hsp70 gain-of-function effects. We utilized the fruit fly, *Drosophila melanogaster*, which offers well-developed genetic approaches for synaptic physiology. Recently developed techniques for *Drosophila* also allow quantification of steady-state $[Ca^{2+}]_i$ at rest and during stimulus trains using the ratiometric fluorophore Fura-dextran (Macleod et al. 2002, 2003).

We examined the thermosensitivity of calcium regulation in nerve terminals at rest and during stimulation to determine the effects of a prior heat shock and genetically altered Hsp70 expression. Both treatments revealed enhanced thermal limits (thermotolerance) of calcium regulation and synaptic transmission. The results support the hypothesis that mechanisms involving Hsp70, which regulate $[Ca^{2+}]_i$, are major determinants of the thermotolerance of synaptic transmission.

METHODS

Fly stocks

All the flies were raised on standard cornmeal medium at 25°C and 60–70% relative humidity. Early wandering third-instar larvae were selected for the experiments.

To increase the expression of Hsp70 in motor neurons, we used larvae of a UAS-Hsp70 line (UAS-Hsp70-4.3/+) constructed in this laboratory (Xiao et al. 2007). In this transgenic line, Hsp70Ab gene coding sequence (CDS), one of six hsp70 alleles in *Drosophila*, was

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inserted into a GAL4-responsive vector, pINDY5, which contained a c-myc tag encoding for a 10 amino acid sequence (EQKLISEEDL). This allowed epitope tagging of the Hsp70Ab CDS. Motor neuron-specific expression of Hsp70 was carried out using theUAS/Gal4 binary system (Brand and Perrimon 1993) by crossing to the GAL4 driver D42 (Parkes et al. 1998).

Other *Drosophila* lines used included Canton-S for the heat shock and wild-type control, as well as the genetic background controls w^{1118}, D42-Gal4, and UAS-Hsp70 for the Hsp70 motor-neuron-specific expression experiments.

Experimental procedures

Heat shock was administered by placing intact *Drosophila* larvae in a sealed petri dish with moist paper; the dish was then placed for 1 h in an oven set at 36°C. Experiments were carried out following 2–4 h of recovery at room temperature (~20°C), except for records of the experiment used in Fig. 4 which were taken between 2–6 h of recovery.

For physiological experiments, the dissection was carried out in Schneider's Insect Medium (Sigma-Aldrich) to minimize contractions and reduce chance of damage during dissection. The larval surface was cut up the dorsal midline and pinned open; the internal organs were then removed, leaving the nervous system and body wall muscles intact. Physiological recordings were obtained from preparations that were continually superfused with HL6 solution [(Macleod et al. 2002) containing 0.5 mM Ca^{2+}. HL6 contained (in 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 BES, 80.0 trehalose·$2H_20$, 5.7 L-alanine, 2.0 L-arginine·HCl, 14.5 glycine, 11.0 L-histidine, 1.7 L-methionine, 13.0 L-proline, 2.3 L-serine, L-threonine, 1.4 L-tyrosine, 1.0 L-valine, 1.0 Trolox, and 0.0001 TPEN]. L-glutamate (7 mM) was added to the solution to desensitize glutamate receptors to prevent muscle contractions during stimulation and imaging of Ca^{2+} (Macleod et al. 2002). Tetrodotoxin (1 μM; Sigma-Aldrich) was used to block voltage-gated sodium channels.

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, which is determined by both the stress intensity and duration. For calcium studies, which employed Fura-dextran, images were taken for 200 ms, once every 5 s, throughout a 20-s period at rest, over 20 s of 30-Hz stimulation, and then over a 20-s period immediately following the cessation of stimulation. Electrophysiological experiments were run using the same protocol as in the calcium imaging experiments with two exceptions: no glutamate was put in the salin; 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 rapidly increasing temperature ramp results in much higher failure temperatures than more slowly increasing temperature ramps (Klose et al. 2004). Thus 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 the ramp was kept the same for 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 1b (large, tonic) nerve terminals were obtained by loading the calcium indicator Fura-dextran (10 kD; 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 340 ± 5 and 380 ± 5 nm band-pass filters on a rapidly switching filter wheel (Omega Optical, Brattleboro, VT). Measurments of fluorescence in response to excitation at these two wavelengths allow ratiometric calculation of intra-terminal $[Ca^{2+}]$.

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. 1996). 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).

Calcium kinetics at higher temporal resolution were examined using the more sensitive nonratiometric indicator Oregon Green 488 Bapta-dextran (OGB). Line scanning through individual type 1b nerve terminals was done using a BioRad 600 confocal scan-head (BioRad MRC-600 software; BioRad, Mississauga, Ontario, Canada) on a Nikon upright microscope (Optiphot-2) with a ×40 Olympus water-immersion objective (0.7 NA). Images were acquired using a BHS filter set (exciter filter-488 nm DF10; emission filter-OG 515 nm LP; dichroic reflector-510 nm LP). The gain of the photo-multiplier was maximized while the pinhole was opened to its maximum aperture, resulting in an acceptable level of fluorescence when the output of the argon ion laser (low power) was attenuated to 0.5% by neutral density filters. A green light-emitting diode, placed within the scan-head, was lit briefly (4 ms) to mark the beginning of the stimulus train (Bao et al. 2005; Macleod et al. 2004). Line scan rates for *X–Y* and *Y*-time scans were 4 ms per line. Pixel values of scanned images were saved in PIC files with 8-bit depth. A look-up table was used to represent pixel values in an 8-bit range. Fluorescence (F) is reported in arbitrary units with background subtracted. Nerves were stimulated at 30 Hz with 0.5 mM external calcium concentration. Data collected in HS and NHS larvae with the genetically expressed calcium indicator GCaMP (Wang et al. 2003) agreed with OGB data, indicating that the loading procedure had no apparent effect on results (data not shown). OGB has a better signal-to-noise ratio and better temporal resolution than the genetically coded indicator (Reiff 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 or within lines of a line-scan image. 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. This value was subtracted numerically from the average pixel value of a region or line containing the fluorescent element of interest to generate the value F. $\Delta F/F$ is defined as the change in F during stimulation, relative to F prior to nerve stimulation.

Stimulation protocol

In both electrophysiological and calcium imaging experiments, nerves were stimulated with a duration of 0.5 ms and just superthreshold stimulus intensity (4–6 V). When responses were lost at moderate temperatures (≤37°C), negative pressure applied to the suction electrode or a small increase in stimulus intensity of 0.5–2 V restored evoked calcium responses in all cases. At high temperatures, following the failure of electrical or fluorescence responses, the stimulus intensity was increased (20–100 V) to electrotonically stimulate voltage-activated calcium channels. Electrotonic stimulation has been used successfully to directly recruit voltage-activated calcium chan-

nels, thus bypassing the need of sodium channels activation (Bronk et al. 2001).

Statistical analysis

Significance of $P < 0.01$ (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 ± SE.

RESULTS

Hyperthermia-induced disruption of nerve terminal function

To better understand calcium regulation at high temperatures, we recorded fluorescence from individual type 1b (big glutamatergic) boutons loaded with OGB every 4 ms during 30-Hz stimulation at 41°C. At this temperature, neuromuscular transmission in untreated isolated preparations failed within minutes, and presynaptic calcium responses exhibited intermittent failures in three of six preparations (Fig. 1*Ai*). The occasional inability to elicit a presynaptic calcium deflection may be the result of intermittent failure of nerve action potentials or the inability to recruit voltage-activated calcium channels. An intracellular postsynaptic recording under the same condition also reveals intermittent failures of excitatory junctional potentials (Fig. 1*Bi*).

Following the onset of intermittent failure at 41°C and prior to the complete failure of synaptic transmission, spontaneous calcium deflections were observed in two of six preparations (Fig. 1*Ci*). A substantial increase in spontaneous postsynaptic electrical activity occurred prior to failure of evoked responses as seen midway through the recording shown in Fig. 1*Di.* At lower temperatures, events recorded at individual type 1b boutons were generally ≤0.1 Hz (Karunanithi et al. 1999).

In contrast with untreated preparations, HS-treated preparations (1 h at 36°C) did not show the loss of evoked responses. Similarily, in contrast with untreated preparations, HS preparations did not gain spontaneous events at 41°C (Fig. 1, *Aii, Bii, Cii,* and *Dii*). Thus there is a HS-mediated improvement in synaptic function at high temperatures occurring in tandem with an extension of calcium homeostasis.

Effect of HS on calcium kinetics

To investigate HS effects on calcium regulation, we compared kinetics and magnitude of stimulus-evoked calcium signals in HS and NHS control preparations at several test temperatures. Using line scanning with the confocal micro-

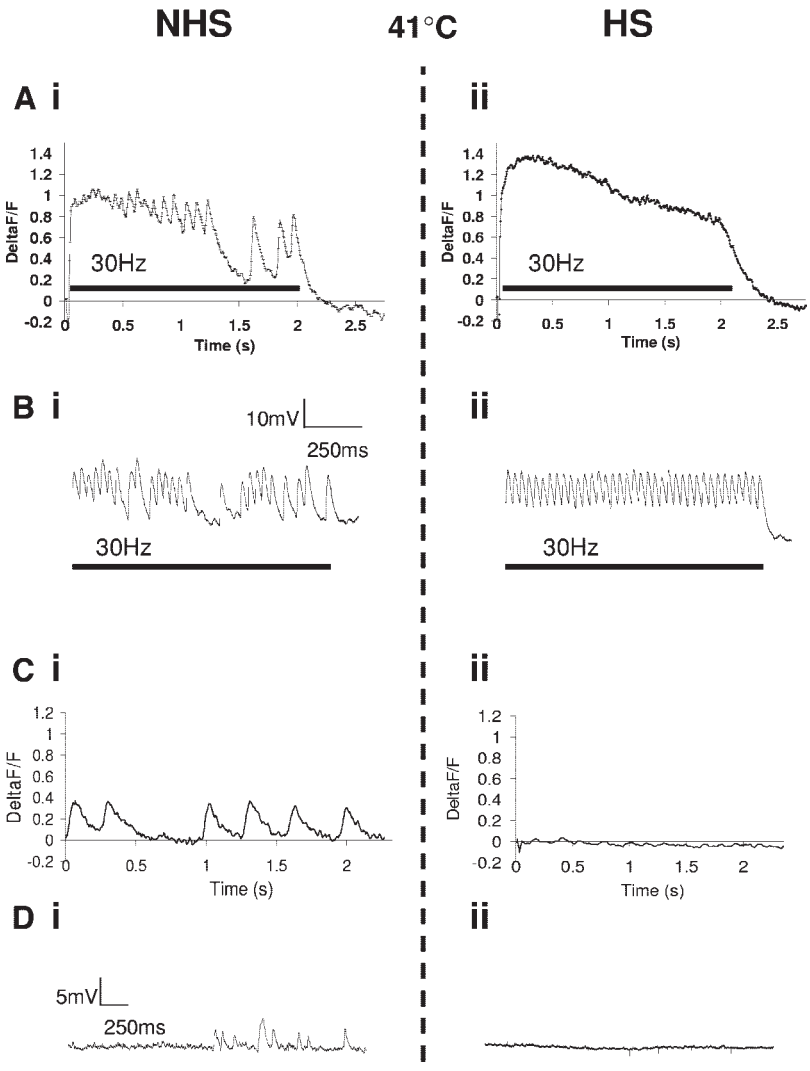

FIG. 1. Failure at high temperature of both synaptic transmission and evoked Ca^{2+} responses. *A, i*: Oregon Green 488 Bapta-dextran (OGB) fluorescence in a non-heat-shocked (NHS) nerve terminal at 41°C. This recording was taken following the 20-s stimulation burst carried out at each temperature in all of the stepwise temperature ramp experiments. Failures in evoked calcium responses increase progressively with continued stimulation. *ii*: HS preparation exhibits no failures. *B, i*: an electrophysiological trace during stimulation at 41°C also revealed intermittent failures to respond to stimulation. *ii*: HS preparation exhibits no failures. *C, i*: at 41°C in NHS terminals, spontaneous calcium deflections were observed. *ii*: HS preparation exhibits no spontaneous calcium deflections. *D, i*: intracellular recording revealed the onset of intense spontaneous activity, just prior to complete failure of synaptic transmission. *ii*: HS preparation exhibits no increase in spontaneous release.

scope (Fig. 2*A*), we measured the rise time (20–80%), decay time constant, and maximum change in OGB's calcium-induced fluorescence (max $\Delta F/F$) during 30-Hz stimulation according to Macleod et al. (2002, 2003). "Greater plateau concentrations of calcium can result in larger deviations of decay rates from single-exponential behavior with faster initial decays (Tank et al. 1995), thus we derived decay time constants from the single-exponential tail end component of the decay so that the different starting points would not skew the results." Line scanning of nerve terminals at RmT (~22°C) revealed no differences in $\Delta F/F$ between HS ($n = 6$) and NHS ($n = 6$) terminals during 1 s of stimulation (Fig. 2*B*). At 39°C, OGB fluorescence in nerve terminals during 2 s of 30-Hz stimulation increased for the first 200 ms but then decayed during the ongoing stimulus train (Fig. 2*C*). At 39°C, Ca^{2+} signals of HS terminals have a shorter rise time ($P > 0.02$) and a higher max $\Delta F/F$ ($P > 0.01$) than those of NHS terminals although the decay time constant was unaffected (Fig. 2*D, i–iii*). These data show that a HS preconditioning treatment has no effect on the calcium entry into the cytosol at RmT but enhances it at 39°C, as evidenced by its larger amplitude and faster rise.

At 43°C, stimulus-evoked calcium responses comparable to those at 41°C could still be elicited in all HS terminals, whereas only one NHS preparation responded to stimulation in a small, unsustained manner (Fig. 3*Ai*).

Progressively increasing the stimulus intensity, from threshold (~5 V with 0.5-ms duration) to a maximum 100 V to electrotonically recruit voltage-activated calcium channels, occasionally elicited a small calcium response in NHS preparations at 43°C that did not decay following the cessation of stimulation (Fig. 3*Aii*). The threshold to electrotonically evoke

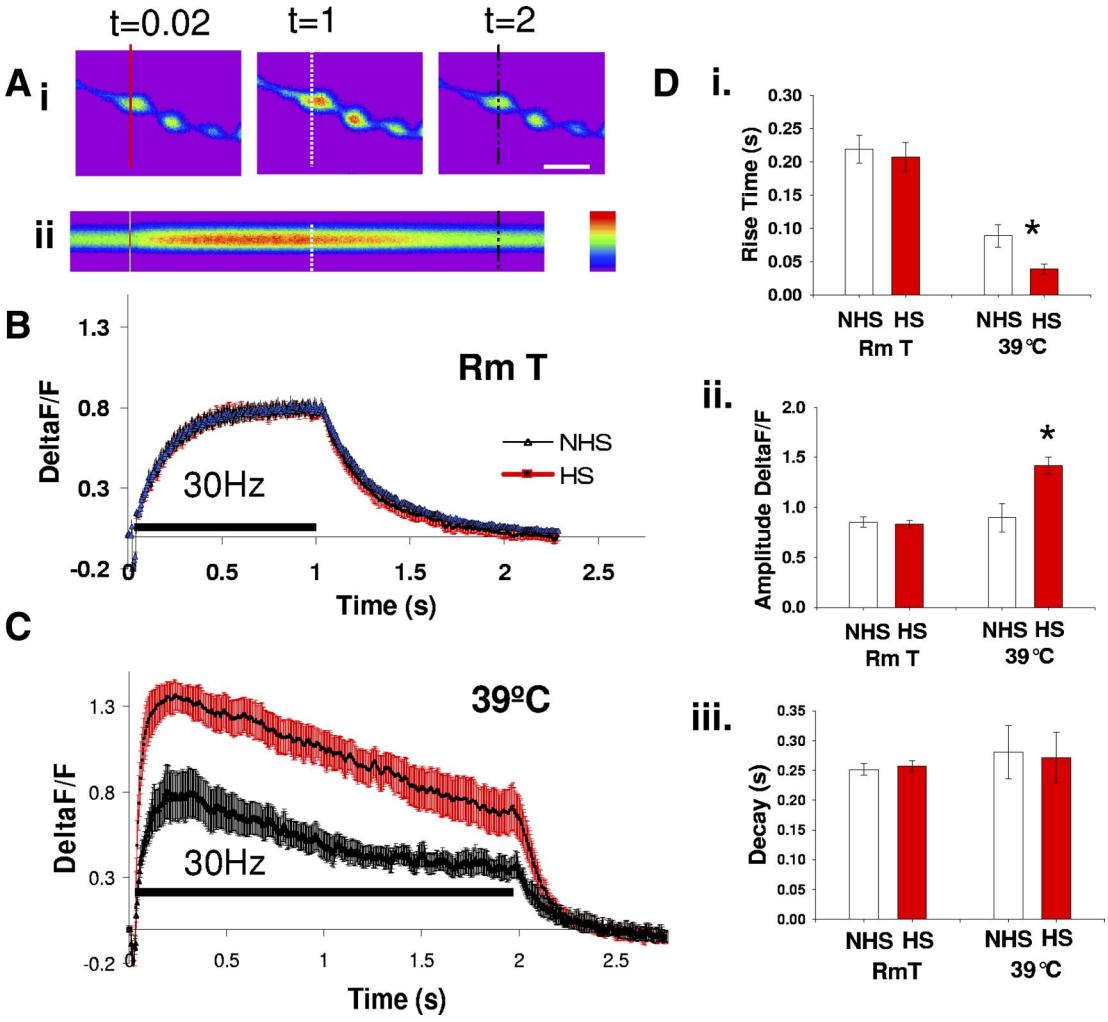

FIG. 2. Heat shock protects kinetics and amplitude of Ca^{2+} response. Calcium kinetics were assessed in type 1b nerve terminals, using the OGB-dextran in NHS and HS preparations after 2–4 h of RmT recovery. Asterisk, HS values ($n = 6$) are significantly different from NHS ($n = 6$) values. *A, i*: OGB-loaded terminals before, during, and after stimulation. Colored lines indicate location of the scan through the nerve terminal that corresponds to the line scan below. *Ii*: fluorescence line scan from above terminal. *B*: rise and decay of *F* during stimulation, relative to *F* prior to nerve stimulation (*F*/*F*) in HS and NHS terminals at RmT. *C, i*: OGB fluorescence at 39°C in HS (red) and NHS (black) nerve terminals during 2 s of 30-Hz stimulation peaks and then progressively decreases until a steady-state level is reached near the end of 2 s of stimulation. *D*: fast calcium kinetics at both RmT and 39°C. *i*: rise time of HS terminals was 38.5 ± 7.5 ms and was significantly faster than NHS terminals at 88.4 ± 16.5 ms ($P = 0.02$); *ii*: the max $\Delta F/F$ of HS terminals was 1.42 ± 0.09 and was higher than in NHS terminals, 0.90 ± 0.14 ($P = 0.01$); *iii*: no difference in decay time was observed in HS terminals, 27.3 ± 0.4 ms, when compared with NHS terminals, 28.1 ± 0.5 ms ($P > 0.01$). *i–iii* show no difference between HS and NHS at RmT. The rise time was the duration measured from 20% to 80% of the calcium response amplitude. Decay times were determined with nonlinear regression analysis using the equation $y = y_0 + ae^{(-bx)}$ (Sigmaplot 9 software). Only the single-exponential component was used in the analysis.

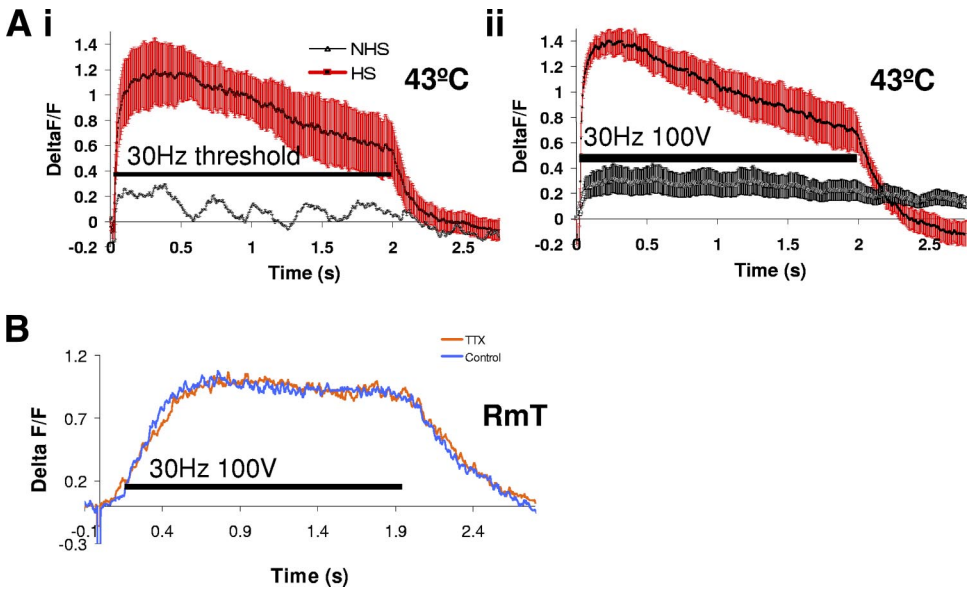

FIG. 3. Electrotonic stimulation cannot fully recover evoked responses at high temperature. *A, i*: At 43°C HS-treated terminals continued to respond for the duration of the 2-s stimulation. Only 1 of 6 NHS terminals responded to stimulation. *ii*: increasing the stimulus intensity by more than an order of magnitude (20–100 V) revealed a small response in NHS terminals. Following the cessation of stimulation in NHS terminals, the fluorescence did not return to prestimulus levels, and no further increases in fluorescence could be stimulated, regardless of stimulus intensity. These recordings were taken as close in temporal proximity as possible to those of the previous recordings shown in *Ai*. *B*: electrotonic stimulation of NHS boutons, in the presence of tetrodotoxin 1 μM and reduced external Na^+, does not decrease stimulus-induced Ca^{2+} response amplitude.

responses was between 20 and 45 V ($n = 6$). Electrotonic stimulation has been used successfully to directly recruit voltage-activated calcium channels, thus bypassing effects of action potentials (Bronk et al. 2001). No further presynaptic calcium responses could be elicited by 30-Hz nerve stimulation. Electrotonic stimulation of HS terminals elicited responses that were comparable in rise, amplitude, and recovery to those evoked with low-intensity levels at the same temperature. This revealed that electrotonic stimulation had no apparent detrimental effect on calcium regulation of HS nerve terminals nor did it alleviate the activity-dependent depression of their calcium responses. In addition, when the Na^+ in the bathing solution was replaced with *N*-methyl-D-glucosamine (NMDG) and TTX was added, response amplitude during electrotonic stimulation at room temperature was not reduced (Fig. 3*B*) even with multiple consecutive bouts of 30-Hz stimulation. Electrotonic stimulation can successfully circumvent the loss of action potentials without disrupting calcium channel activity.

The loss of stimulus-evoked calcium responses occurred at temperatures where synaptic transmission also failed. Failure of synaptic transmission in NHS preparations occurred at 41.5 ± 0.3°C ($n = 8$). Following the failure of synaptic transmission in NHS preparations, electrotonic stimulation could elicit a small transient postsynaptic response in some preparations. This transient recovery of the postsynaptic response paralleled the recovery of the calcium response observed with the same elevation in stimulus intensity (Fig. 3). The data reveal a strong correlation between failure to elicit a rise in calcium fluorescence and the failure of synaptic transmission, suggesting a functional link.

Effect of a prior heat shock on resting calcium concentration

The hyperthermia-induced increase in spontaneous release at elevated temperatures prior to failure of synaptic transmission (Fig. 1*D*) suggests elevated resting calcium concentration in the presynaptic terminal. To measure the absolute cytosolic-free calcium concentration in nerve terminals, we recorded Fura fluorescence at 340 and 380 nm. At each temperature, ratiometric recordings were taken every 5 s for 20 s before, 20 s during, and 20 s after 30-Hz stimulation. We examined the effects of HS and Hsp70 expression on the thermosensitivity of cytosolic calcium concentration.

The normal resting calcium concentration ($[Ca^{2+}]_r$) at RmT in NHS nerve terminals was 39.2 ± 2.6 nM ($n = 18$; Fig. 4, *B* and *C*). $[Ca^{2+}]_r$ recorded at RmT following heat shock treatment (1 h at 36°C) was significantly elevated but gradually reverted back to NHS levels over a 6-h recovery period at RmT (Fig. 4*B*). The average $[Ca^{2+}]_r$ following 2–4 h of post-HS recovery at RmT was 58.7 ± 3.0 nM ($n = 18$; Fig. 4*C*).

The thermosensitivity of calcium regulation was assessed during a stepwise temperature ramp. The $[Ca^{2+}]_r$ was an average of four recordings at each temperature taken every 5 s for 20 s just prior to 20 s of 30-Hz stimulation. The $[Ca^{2+}]_r$ increased with increasing temperature although the rise was significantly less following HS treatment (Fig. 4*C*). HS-treated preparations showed $[Ca^{2+}]_r$ higher than control at low temperatures but, conversely, lower than control at high temperatures >37°C. Therefore HS preparations were able to maintain $[Ca^{2+}]_r$ closer to normal than NHS preparations at high temperatures.

Effect of 30-Hz stimulation and heat shock on nerve terminal function

During 30-Hz stimulation at RmT, the stimulus-evoked calcium concentration ($[Ca^{2+}]_s$) reached a plateau of 134.1 ± 8.8 nM in NHS control terminals ($n = 18$), while the $[Ca^{2+}]_s$ of HS-treated terminals ($n = 18$) was significantly higher: 161.6 ± 9.5 nM (Fig. 5*A*). All P values are <0.05 unless otherwise stated. The amplitudes of the evoked Ca^{2+} responses were not different because resting Ca^{2+} levels in the HS terminals were higher (see also Fig. 2*B*).

The amplitude of these responses (ΔCa^{2+}_{rise}) is the difference between $[Ca^{2+}]$ before and during stimulation ($[Ca^{2+}]_s - [Ca^{2+}]_r = \Delta Ca^{2+}_{rise}$). This measures the net stimulus-evoked Ca^{2+} build-up in the nerve terminal and can be used as an indicator of Ca^{2+} entry. At RmT, there was no effect of HS on ΔCa^{2+}_{rise}, whereas at higher temperatures (37–43°C), ΔCa^{2+}_{rise}

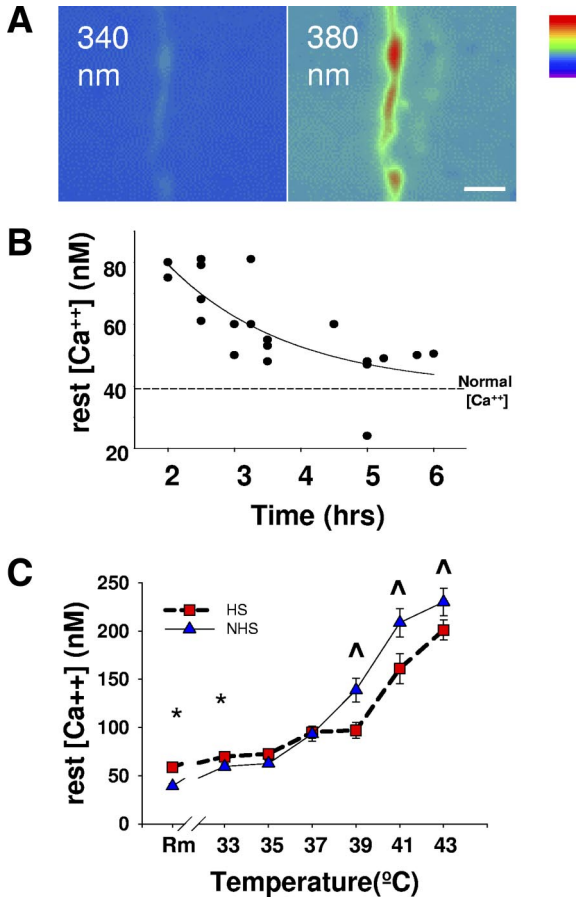

FIG. 4. Heat shock preconditioning alters thermosensitivity of resting calcium concentration *Drosophila* nerve terminals. The $[Ca^{2+}]$ in type 1b nerve terminals was measured using Fura-dextran after 2–4 h of RmT recovery following HS. In this and subsequent figures, error bars represent SE; *, HS values ($n = 18$) significantly greater than NHS values ($n = 18$); ^, NHS values significantly greater than HS values. Measurements were made after 4 min at each temperature. *A*: images of Fura-dextran-loaded terminals acquired through a 530-nm band-pass filter while exposed to either 340- or 380-nm excitation. Scale bar 5 μm. *B*: calcium was measured following the 2-h loading process. HS-induced elevation of $[Ca^{2+}]_r$ recovers in a time-dependent manner at RmT. Control level of $[Ca^{2+}]_r$ shown by dotted line. Elevation in $[Ca^{2+}]_r$ following HS is restored back to NHS levels over a 6-h period at RmT. The superimposed curve is the best fit of the data to an exponential decay of the form $Ca^{2+} = Ca^{2+}0 + ae^{(-b\text{time})}$ with y_0 constrained to 39.2. *C*: the $[Ca^{2+}]_r$ of HS terminals remained elevated above NHS levels after 2–4 h of RmT recovery following the HS treatment. Elevation of temperature led to an increase in $[Ca^{2+}]_r$ in both treatment groups; however, the temperature-induced increase was less dramatic in HS preparations. At high temperatures, the $[Ca^{2+}]_r$ in preparations subjected to a HS preconditioning treatment was less than in NHS control terminals.

in HS terminals was significantly greater than in NHS terminals (Fig. 5, *A–F*). At 41°C, not all NHS terminals responded to low-intensity stimulation of the nerve. At 43°C, Ca^{2+} responses could not be evoked in NHS terminals, whereas in HS terminals, the same stimulus could do so successfully (Fig. 5*E*). A substantial elevation in stimulus intensity was required to evoke a small but detectable response in NHS terminals at 43°C (Fig. 5*F*). Most NHS terminals responded to electrotonic stimulation; however, responses were very small compared with those in HS terminals. In HS preparations, increased stimulus intensity allowed terminals to respond for the entire duration of the stimulus train in all but one preparation (Fig. 5*F*).

The difference between $[Ca^{2+}]$during stimulation and after stimulation (ΔCa^{2+}_{fall}) is a measure of the net calcium clearance following stimulation and can be used as an indicator of the viability of clearance mechanisms. The $[Ca^{2+}]_r$ was measured every 5 s after stimulation for 20 s. At RmT, this was sufficient time for $[Ca^{2+}]_r$ to return to prestimulus resting levels in both HS and NHS terminals (Fig. 5*A*). Thus the calcium clearance mechanisms could remove the full calcium load that had built up at RmT. However, at 37, 39, and 43°C, the ΔCa^{2+}_{fall} in HS terminals was significantly larger than in NHS control terminals (Fig. 5, *C–F*), indicating that after HS treatment the clearance mechanisms removed cytoplasmic calcium more completely at these higher temperatures.

The difference between $[Ca^{2+}]_r$ before and after stimulation (ΔCa^{2+}_{rest}) assesses the nerve terminal's ability to retain calcium homeostasis following stimulation. At low temperatures, there was no difference in ΔCa^{2+}_{rest} between HS and NHS nerve terminals (Fig. 5*A*). At 37°C, the $[Ca^{2+}]_r$ did not return to prestimulus levels in NHS terminals but did in HS terminals; thus ΔCa^{2+}_{rest} was significantly greater in NHS terminals (Fig. 5*B*). This revealed the untreated preparation's impaired ability to restore normal resting Ca^{2+}. At 39, 41, and 43°C, the $[Ca^{2+}]_r$ following successfully evoked responses did not recover rapidly to prestimulus resting levels in either treatment group, i.e., ΔCa^{2+}_{rest} was significantly greater than zero (Fig. 5, *C–E*). In addition, temperature ramp experiments done without stimulation showed no difference between NHS and HS terminals >33°C (data not shown). Thus stimulation at high temperatures had a detrimental effect on $[Ca^{2+}]_r$, which is attenuated by HS.

The average values and standard errors of $[Ca^{2+}]_r$, $[Ca^{2+}]_s$, ΔCa^{2+}_{rise}, ΔCa^{2+}_{fall}, and ΔCa^{2+}_{rest} are shown for RmT, 37, 39, 41, and 43°C in Table 1.

Role of Hsp70 expression on nerve terminal thermotolerance

Previous research has shown that a prior HS can increase synaptic thermotolerance and that Hsp70 overexpression in genetically engineered *Drosophila* lines can further enhance the level of synaptic thermoprotection (Karunanithi et al. 1999, 2002). However, research examining the locomotor behavior of larvae has found that ubiquitous Hsp70 overexpression slightly tempers the level of thermoprotection (Klose et al. 2005), whereas motorneuron-specific upregulation of Hsp70 enhances thermoprotection of this behavior (Xiao et al. 2007). To obtain a clearer view of Hsp70's role in synaptic function, we examined the thermosensitivity of calcium regulation in nerve terminals of larvae expressing Hsp70 specifically targeted to the motor neurons using a UAS Hsp70 construct (Xiao et al. 2007) and a D42 motor-neuron-specific Gal4 driver (Parkes et al. 1998). Two different motorneuron-specific drivers, OK6 and D42, revealed similar protective effects on locomotion, ruling out unidentified secondary background effects (Xiao et al. 2007); thus we used only D42 in our nerve terminal calcium imaging experiments. Immunohistochemistry confirmed expression of transgene Hsp70 in nerve terminals using the D42 driver, while genetic controls of the transgene and driver revealed no effects on Hsp70 expression or thermotolerance of locomotion (Xiao et al. 2007). Locomotory behavior in larvae expressing Hsp70 in motor neurons was indistinguishable from wild type, suggesting no deleterious effects of transgene expres-

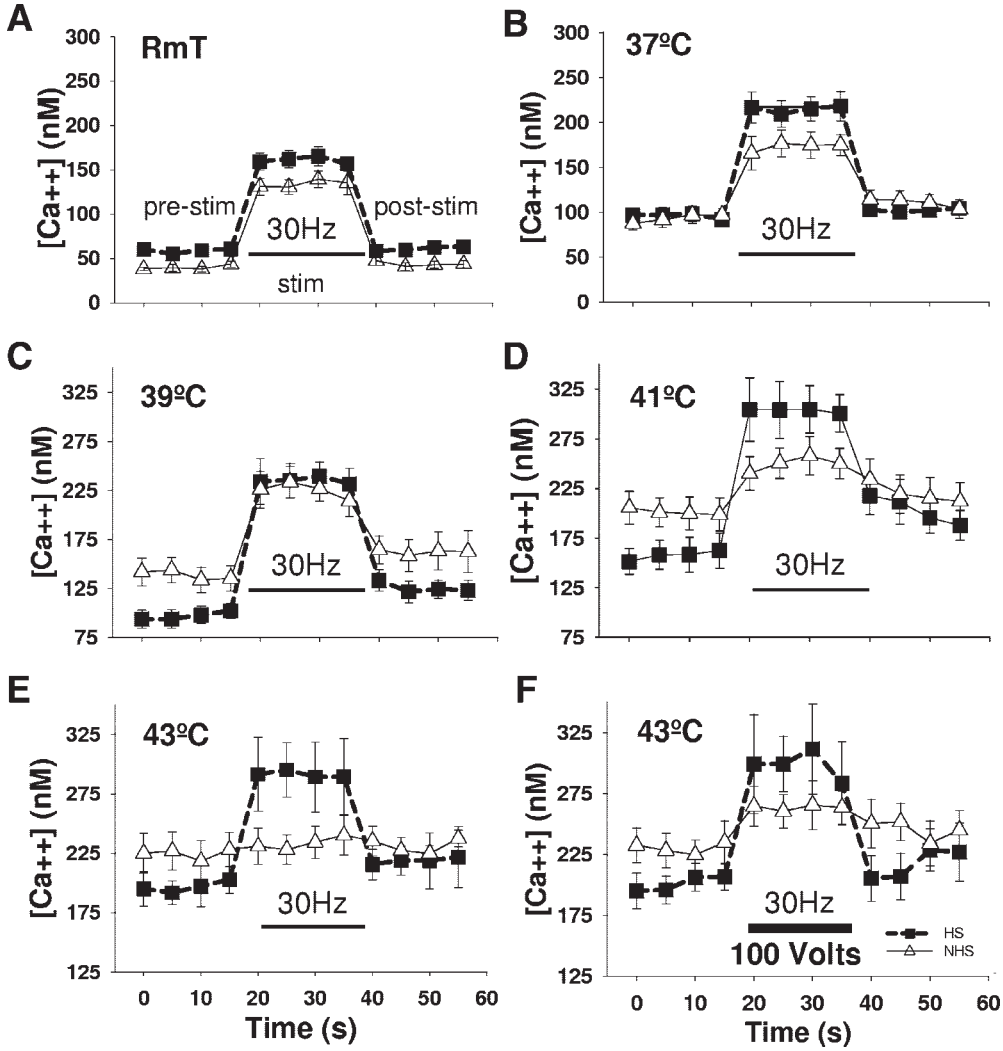

FIG. 5. Heat shock preconditioning alters stimulus-evoked Ca^{2+} levels in *Drosophila* nerve terminals. The $[Ca^{2+}]$ in type 1b nerve terminals was measured using the ratiometric Ca^{2+} indicator Fura-dextran in NHS and HS preparations after 2–4 h of recovery at RmT. Fluorescence measurements reveal elevated $[Ca^{2+}]$ in HS preparations before, during, and after 20 s of 30-Hz stimulation. For significance indicators see Table 1. *A*: at RmT, the ΔCa^{2+}_{rise}, ΔCa^{2+}_{fall}, and ΔCa^{2+}_{rest} were not different. *B*: at 37°C, the ΔCa^{2+}_{rise} and ΔCa^{2+}_{fall} were not different, but the ΔCa^{2+}_{rest} was significantly higher in NHS terminals. *C*: at 39°C, both ΔCa^{2+}_{rise} and ΔCa^{2+}_{fall} are significantly greater in HS terminals. *D*: at 41°C, ΔCa^{2+}_{rise}, ΔCa^{2+}_{fall} and ΔCa^{2+}_{rest} are significantly greater in HS terminals. *E*: at 43°C, ΔCa^{2+}_{rise}, ΔCa^{2+}_{fall} and ΔCa^{2+}_{rest} are significantly greater in HS terminals. Threshold stimulation did not elicit an evoked calcium response in NHS control terminals but did in HS terminals. *F*: at 43°C, some NHS preparations did respond to stimulation when the intensity was increased by an order of magnitude over threshold (20–100 V) to electrotonically stimulate voltage-activated Ca^{2+}-channels. HS preparations responded to electrotonic stimulation. Both ΔCa^{2+}_{rise} and ΔCa^{2+}_{fall} are significantly greater in HS terminals.

sion at room temperature (Xiao et al. 2007). Because no heat shock was required for Hsp70 expression, these experiments allowed us to examine its effects more directly without the confounding effects of the stress conferred by the heat shock treatment or of upregulation of other chaperones by heat shock.

At RmT, calcium regulation was not altered by Hsp70 expression in the UAS Hsp70 × Gal4D42 line ($n = 8$); however, at 39 and 41°C (Fig. 6, *B* and *C*), Hsp70 expression significantly lowered $[Ca^{2+}]_r$ in comparison with controls ($n = 10$). At 43°C, Ca^{2+} responses could be evoked in Hsp70-expressing terminals, while in the genetic control terminals only electrotonic stimulation could evoke a response (Fig. 6, *D* and *E*). Thermoprotection of $[Ca^{2+}]_r$ in nerve terminals expressing Hsp70 ($n = 6$) coincided with thermoprotection of synaptic transmission. Hsp70 expression in motor neurons significantly elevated the temperature at which synaptic transmission failed by 1.6°C above controls ($n = 6$; Fig. 6*F*). This revealed a role for Hsp70 in thermoprotection of calcium regulation and confirmed its role in thermoprotection of synaptic transmission.

DISCUSSION

We examined the role of calcium regulation in maintenance of synaptic transmission at high temperatures. As temperature increases, the resting free Ca^{2+} concentration increases. At stressfully high temperatures, electrical stimulation exacerbates the $[Ca^{2+}]_r$ increases, and both synaptic transmission and stimulus-evoked presynaptic Ca^{2+} responses eventually fail. Both HS preconditioning and Hsp70 expression in motor neurons, which have been shown to thermoprotect synaptic transmission, motor pattern generation, and locomotor behavior (Karunanithi et al. 1999; Klose et al. 2005; Xiao et al. 2007), attenuated the detrimental increase in $[Ca^{2+}]_r$ and maintained stimulus-evoked Ca^{2+}-responses. In thermotolerant ter-

TABLE 1. *Calcium values in Fura-dextran-loaded nerve terminals*

	RmT		37°C		39°C		41°C		43°C		43°C electro	
	NHS	HS	NHS	HS	NHS	HS	NHS	HS	NHS	HS	NHS	HS
ΔCa^{2+}_{rest}	4.3 ± 1.5	2.3 ± 3.0	**18.1 ± 3.8**	6.5 ± 3.4	22.5 ± 6.8	27.5 ± 6.2	21.1 ± 5.6	**44.6 ± 9.3**	6.6 ± 3.1	**23.0 ± 9.5**	15.2 ± 15.0	17.0 ± 15.5
ΔCa^{2+}_{rise}	94.9 ± 7.9	102.9 ± 8.0	80.9 ± 10.3	**117.5 ± 11.2**	86.3 ± 10.7	**138.4 ± 14.0**	53.5 ± 14.1	**142.6 ± 20.0**	6.2 ± 3.9	**94.5 ± 13.1**	33.4 ± 14.4	**97.33 ± 29.7**
ΔCa^{2+}_{fall}	90.6 ± 8.2	100.5 ± 7.5	62.7 ± 9.1	111.1 ± 13.1	65.6 ± 11.8	**110.5 ± 14.6**	37.0 ± 11.1	**97.8 ± 22.0**	−0.4 ± 4.2	**67.3 ± 8.2**	18.2 ± 9.3	**80.3 ± 19.2**

Results are the average (±SE) of 18 preparations in both HS and NHS groups. The resting and stimulus-induced steady-state values of each preparation is the average of four consecutive recordings made 5 s apart. Changes in calcium levels resulting from stimulation were calculated from the averaged steady-state values for each preparation. Bold numbers indicate the significantly higher value when comparing heat shock (HS) and non-HS (NHS) groups at each temperature. Only RmT and 37–43°C data shown due to space constraints.

minals, lower $[Ca^{2+}]_r$ at high temperatures coincided with maintenance of evoked Ca^{2+} responses. Our preliminary hypothesis is that heat-shock-induced mechanisms including actions of upregulated Hsp70 stabilize presynaptic calcium regulation, protecting synaptic transmission at high temperatures.

Heat-shock-induced increase of resting calcium at room temperature

Heat shock preconditioning resulted in a small but long-lasting elevation in $[Ca^{2+}]_r$ at room temperature This may seem counterintuitive because the HS response protects synaptic transmission and large elevations in cytosolic Ca^{2+} can damage cellular function (Budd 1998; Kiang et al. 1998). However, small elevations in $[Ca^{2+}]_r$ are necessary for some forms of synaptic plasticity (Zucker and Regehr 2002), and heat shock protein synthesis is likely Ca^{2+} dependent (Kiang et al. 1999, 2000; Taylor et al. 2007). The small HS-induced elevation in $[Ca^{2+}]_r$ may initiate thermoprotective mechanisms. Restoration of $[Ca^{2+}]_r$ in *Drosophila* nerve terminals following HS requires 6 h at RmT; a similar time is required for both HS-induced elevation of Hsp70 and HS-induced thermoprotection of synaptic transmission to revert to normal (Karunanithi et al. 1999).

Thermoprotection of evoked synaptic responses

HS-treated terminals exhibited faster rise times and greater maximum $\Delta F/F$ of Ca^{2+} responses at high temperatures, suggesting more calcium entry during stimulation. We based our interpretations on a single-compartment model of Ca^{2+} described by Tank et al. (1995), appropriate for the *Drosophila* bouton because of its small size and the fast equilibration of Ca^{2+} by diffusion within the bouton. At 39°C, evoked response amplitude in all nerve terminals increased for the first 200 ms of stimulation then progressively decreased. The activity-dependent reduction in amplitude is also observed during electrotonic stimulation, when the effects of Na^+ channels are bypassed, suggesting direct effects of hyperthermia on Ca^{2+} regulation. One explanation is that the elevated $[Ca^{2+}]_r$ and the build-up of Ca^{2+} during stimulation act together to inhibit Ca^{2+} entry. The greater influx of Ca^{2+} observed in HS terminals at high temperatures could then be explained by the lower $[Ca^{2+}]_r$, allowing for greater Ca^{2+} entry before negative feedback mechanisms act to limit evoked responses. Alternatively, disruption of calcium-induced calcium release from internal stores could account for the reduced response amplitudes. One study suggests that during 30-Hz stimulation internal release of Ca^{2+} stores may account for ≤2/3 of the presynaptic Ca^{2+} response amplitude (Kuromi and Kidokoro 2002). In this scenario, hyperthermia would compromise Ca^{2+} response amplitude by disrupting either ryanodine receptor-mediated release of Ca^{2+} or SERCA-mediated filling of ER stores.

Following failure in some preparations, a small Ca^{2+} response could be evoked with electrotonic stimulation; however, the terminal could not clear the calcium once stimulation had ceased and subsequent responses could not be evoked. Postsynaptic potential responses also recovered under the same conditions, but they too became quickly unresponsive. Thus the vesicle release machinery and postsynaptic responsiveness to transmitter were still functional when Ca^{2+} responses failed.

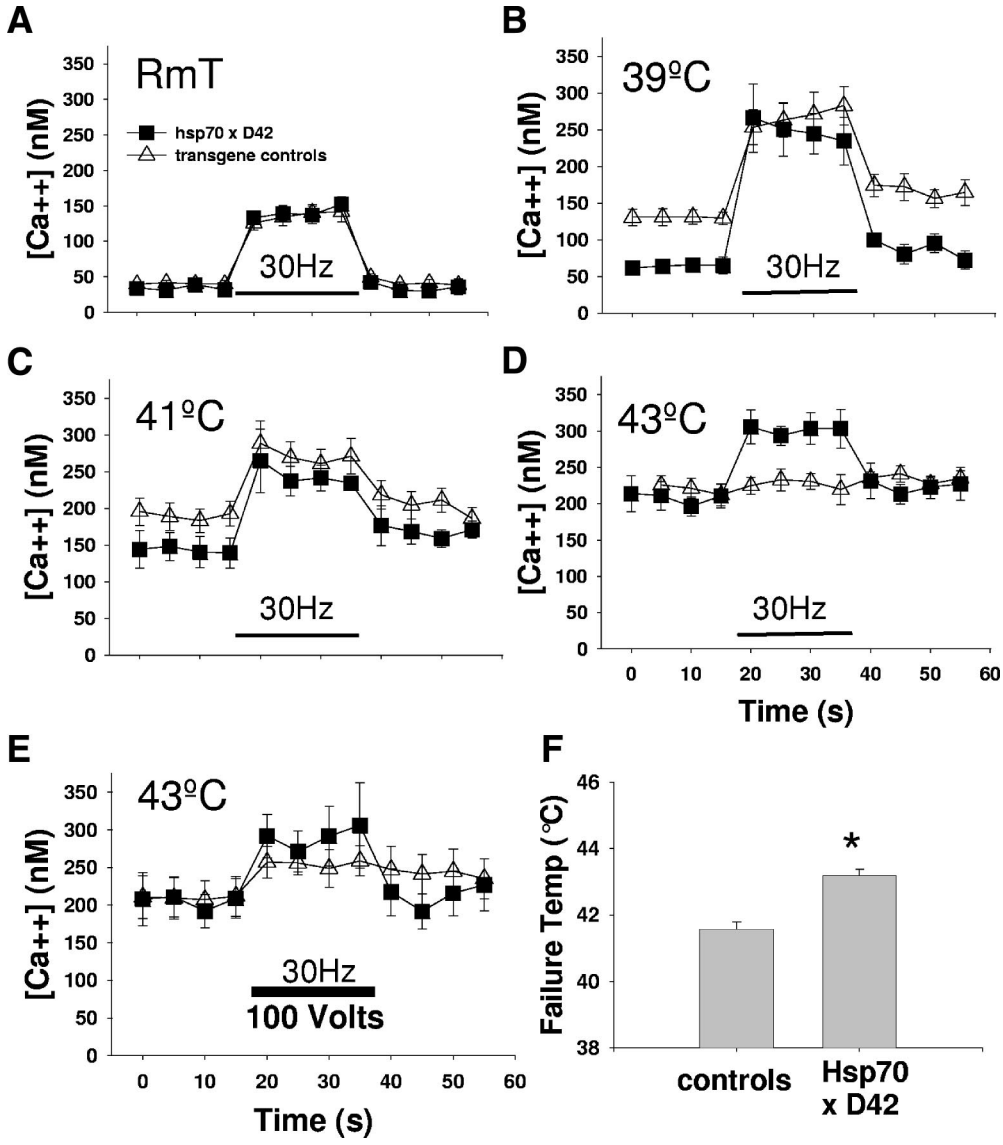

FIG. 6. Effect of transgene Hsp70 expression on thermotolerance of synaptic transmission and resting calcium concentration. Data for the 2 controls, Gal4 control ($n = 5$, progeny from cross between w^{1118} x w^{1118}; D42) and the UAS control ($n = 5$, progeny from w^{1118} x w^{1118}; UAS-Hsp70), were not statistically different from each other and were therefore pooled together. *A*: at RmT, Ca^{2+} regulation of Hsp70 expressing terminals was not different from controls. *B*: at 39°C, the $[Ca^{2+}]_r$ was significantly lower than in control terminals. *C*: at 41°C, the $[Ca^{2+}]_r$ was significantly lower than in control terminals. *D*: at 43°C, the Ca^{2+} responses could only be evoked with threshold stimulation in Hsp70 expressing terminals. *E*: at 43°C, electrotonic stimulation evoked a small Ca^{2+} response in control terminals that did not recover after the stimulus had stopped, whereas in Hsp70 expressing terminals a larger response was evoked and calcium could still be cleared from the cytosol. *F*: the failure temperature of synaptic transmission, assessed by monitoring excitatory junctional potentials, was significantly elevated by motor neuron Hsp70 expression from 41.6 ± 0.2°C in the 2 control lines to 43.2 ± 0.2°C in UAS Hsp70 driven by motor neuron specific Gal4 D42. Significant difference between the control and the Hsp70 expressing boutons (*).

These observations support the idea that disruption of mechanisms responsible for the stimulus-induced accumulation of presynaptic cytosolic Ca^{2+} is responsible for the failure of neuromuscular transmission at high temperatures.

Thermoprotection of resting calcium concentration

Both stimulus-evoked Ca^{2+} responses and postsynaptic potentials fail at ~41°C. Prior to failure, an increase in spontaneous transmitter occurs; this can result from increased presynaptic $[Ca^{2+}]$ (Yoshihara et al. 2000). In control preparations, at this temperature, the terminal is exposed to a prolonged elevation of $[Ca^{2+}]_r$ >200 nM with no respite. The $[Ca^{2+}]_r$ in heat-shock-treated or Hsp70-expressing nerve terminals was maintained <200 nM at 41°C; synaptic function was also maintained, continuing successfully until the temperature reached 43°C when $[Ca^{2+}]_r$ was elevated >200 nM. This suggests that a working range of $[Ca^{2+}]_r$ may exist that, once exceeded, prevents stimulus-evoked Ca^{2+} responses regardless of temperature. Whether stimulus-evoked Ca^{2+}-responses are compromised due to a disruption of calcium influx, disruption of release from internal stores, or a combination of both is unresolved.

High cytosolic Ca^{2+} can negatively modulate Ca^{2+}-channel function (Burgoyne and Weiss 2001; Zuhlke et al. 1999), and the Ca^{2+}-dependent inactivation characteristics of Ca^{2+}-chan-

nels are susceptible to free cytoplasmic calcium levels (Liang et al. 2003). However, 200 nM is likely not sufficient to induce calmodulin-dependent inactivation of Ca^{2+} channels (De Koninck and Schulman 1998) nor calcium-dependent proteases (Pant et al. 1982). The *cac^{ts2}* mutation in the cytoplasmic caboxy tail of the α_1-subunit of *Drosophila* voltage-activated Ca^{2+} channels causes a temperature-sensitive reduction in Ca^{2+} influx. This suggests that temperature-induced disruption of Ca^{2+} influx might contribute to the failure of evoked responses (Macleod et al. 2006). Alternatively, activity-dependent, temperature-sensitive internalization of Ca^{2+} channels, shown in mammalian neuronal cultures (Altier et al. 2005), could be responsible. Although the mechanisms remain to be clarified, sustained elevation of $[Ca^{2+}]_r$ above 200 nM is associated with the disruption of evoked synaptic responses.

Calcium clearance

Pumps that clear cytoplasmic Ca^{2+} during bouts of activity are essential for preventing harmful Ca^{2+} build-up and the ensuing cellular degeneration. At high temperatures, HS-treated terminals exhibit enhanced maintenance of Ca^{2+} clearance. Application of exogenous Hsp70 has been shown to attenuate the thermal inactivation of sarco-endoplasmic reticulum Ca^{+2}-ATPase (SERCA) function in rat muscle (Tupling et al. 2004), suggesting Hsp70 may protect synaptic function through action on calcium pumps. Maintaining SERCA function is required to maintain the ER Ca^{+2} stores necessary for calcium-induced calcium release. Thus thermal inactivation of SERCA in the *Drosophila* nerve terminal would lead to depletion of Ca^{+2} stores and cause a large reduction in the evoked Ca^{2+}-response amplitude as shown at high temperauture. In addition to maintaining calcium clearance, this proposed action of Hsp70 would also aid in maintaining internal calcium stores necessary for normal stimulus-induced Ca^{2+}-response amplitude. The maintained ability of HS terminals to clear calcium at temperatures that are typically nonpermissive reveals a thermoprotection of clearance mechanisms, likely involving Hsp70 action on SERCA function.

Relationship between calcium regulation and synaptic function thermotolerance

At stressfully high temperatures, electrical stimulation exacerbates the $[Ca^{2+}]_r$ increases, and both stimulus-evoked Ca^{2+} signals and synaptic transmission eventually fail. The failure of synaptic function, both evoked Ca^{2+} and evoked postsynaptic responses, is correlated with a $[Ca^{2+}]_r > 200$ nM. Whether the three events are causally related or occurring through independent parallel pathways is presently unknown.

Both heat-shock-treated and Hsp70-expressing terminals reveal thermoprotected $[Ca^{2+}]_r$, evoked calcium responses, and synaptic function, suggesting a possible link between thermotolerance and chaperone-mediated presynaptic calcium regulation.

A recent study by Gong and Golic (2006) revealed that deletion of *hsp70* alleles reduced the thermotolerance level of *Drosophila* behavior, further supporting the idea that Hsp70 levels can modulate thermotolerance of neural function.

A rapid and robust induction of endogenous Hsp70 occurs along the glial sheath of the motor nerves although it was not detected in the glial-free boutons (Brown et al. 1999). However, lack of staining is not conclusive for an absence of Hsp70 in the boutons because the relevant epitope may have been shielded from binding to Hsp70 antibody by interacting proteins. Expression of transgenic Hsp70 in the *Drosophila* lines employed in this study were characterized using immunostaining against the myc peptide incorporated into the Hsp70 transgene to tag the protein, revealing Hsp70 was indeed localized to nerve terminals (Xiao et al. 2007). In mammalian brain tissue, Hsp70 has been shown to associate with lipid rafts (microdomains enriched with cholesterol and sphingolipids containing signal transduction proteins), allowing for interactions with calcium signaling proteins (Chen et al. 2005).

In a *Drosophila* Hsc70 hypomorphic mutant, $[Ca^{2+}]_r$ in nerve terminals is elevated at moderate temperatures and the upper thermal limit of synaptic transmission is reduced (Bronk et al. 2001). Hsc70 is the constitutively expressed version of Hsp70. Similar findings were obtained when the Hsp70 interacting domain of cysteine string protein, which promotes Hsp70's ATPase function, was disrupted (Bronk et al. 2005). Thus loss-of-function studies reveal the importance of Hsp70 in maintaining normal thermotolerance of nerve terminal Ca^{2+} regulation, suggesting a direct role in thermoprotection of synaptic function. In our gain-of-function study, we show that transgenic Hsp70 enhanced the thermotolerance of Ca^{2+} regulation and synaptic transmission supporting our hypothesis.

Together the data suggest that following HS, $[Ca^{2+}]_r$ is better maintained at near-normal levels during hyperthermia through thermoprotection of Ca^{2+} clearance mechanisms, possibly involving Hsp70 and very likely other chaperones acting on mechanisms responsible for calcium homeostasis. Furthermore, we propose that HS-induced thermoprotection of synaptic transmission likely results from greater stability of calcium regulation in presynaptic nerve terminals through chaperone effects involving heat shock proteins such as Hsp70.

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