A Role for the Cytoskeleton in Heat-Shock–Mediated Thermoprotection of Locust Neuromuscular Junctions

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ABSTRACT: A prior hyperthermic stress (heat shock) can induce thermoprotection of neuromuscular transmission in *Locusta migratoria* extensor tibiae muscle measured 4 h after the onset of the heat shock. It is not clear what effect an acute hyperthermic stress may have on the nervous system's ability to tolerate thermal stress, that is, before increased expression of heat-shock proteins. We found that over consecutive thermal stress tests, failure temperature was not altered in either heat-shock or control animals. This suggests that protective mechanisms are not established in the short term (within one hour). Various members of the heat-shock protein family interact with elements of the cytoskeleton. We found that preexposure of the preparation to cytoskeletal stabilizing drugs induced thermoprotection, while preexposure to cytoskeletal disrupting drugs disrupted the ability to confer and maintain thermoprotection. We conclude that thermoprotection relies on a stable cytoskeleton and suggest that members of the heat shock protein family are involved. J Neurobiol 60: 453–462, 2004

***Keywords:* cytoskelton; heat shock-mediated thermoprotection; locust**

INTRODUCTION

Changing environmental conditions can have wide-ranging effects on the operation of the nervous system, and extreme environmental conditions can be deleterious to its function. Poikilotherms are particularly susceptible to large changes in environmental temperatures that can be stressful to the organism. In addition to behavioral responses to cope with environmental stress such as simple avoidance (Robertson et al., 1996), physiological protective mechanisms are initiated under stressful conditions to help animals cope with unavoidable stress. Numerous members of the heat-shock protein family, upregulated at levels of stress for which most other proteins are downregulated (Ritossa, 1996), are believed to be involved in protective mechanisms (Parsell et al., 1993; Sharp et al., 1999; Ohtsuka and Suzuki, 2000). Protection of organisms from the harmful effects of stress can be induced, for a period of time, after a brief initial stressful insult such as an oxidative stress (Dalle-Donne et al., 2001), a heat shock (Whyard et al., 1986; Parsell et al., 1993), or an anoxic shock (Wu et al., 2002). When stress response mechanisms are initiated, they allow individual cells and whole organisms to survive otherwise lethal bouts of stress, a phenomenon referred to as acquired thermotolerance (Feder and Hofmann, 1999).

Prior exposure to a hyperthermic stress not only

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improves an organism's chances of surviving a subsequent stress that would otherwise be lethal, but it also increases the upper temperature limit of nervous system operation. This synaptic thermoprotection phenomenon results in an increase in the upper temperature limit of synaptic transmission in the locust leg muscle (Barclay and Robertson, 2000) and *Drosophila* larval body wall muscle (Barclay and Robertson, 2003). In the locust fast extensor tibiae muscle a stabilization of response amplitude as temperature increases, and an increased ability to recover following the stress are also seen (Barclay and Robertson, 2000).

An abundance of literature exists on the combined effects of stress intensity and the duration of exposure, especially with respect to therapies in the treatment of cancer, for example (Gerner, 1987). Previous experiments using locust muscle to test the thermal limits of neuromuscular transmission have used a 5°C/min temperature ramp stress test, and it is not known if the upper temperature limit of synaptic transmission is significantly altered by the duration of stress exposure, during the stress test itself. It is possible to define critical thermal dosages that depend on the duration of heat exposure as well as its intensity. We used two ramp rates, 5°C/min and 15°C/min, to test for time dependence of hyperthermia-induced failure of synaptic transmission. If the temperature at which synaptic transmission failed were only marginally time dependent and relied primarily on the temperature intensity, it might suggest that the direct physical effects of temperature upon its target, for example, conformational change in a signalling protein such as desensitization of glutamate receptors or inactivation of a calcium channel are responsible for failure. If temperature-induced failure of synaptic transmission is the result of a combination of both the intensity and duration of the stress it would indicate the involvement of a time-dependent process such as the gradual accumulation of protein damage, for example, disruption of cytoskeletal interactions with associated proteins.

Locusts maintained at 45°C for 3 h reveal both acquired thermotolerance (increased survival at high temperatures) and synaptic thermoprotection (increased temperature at which transmission fails). Whether acquired thermotolerance and synaptic thermoprotection are mediated through the same pathways is unknown. Induction of acquired thermotolerance requires a minimum duration of 1.5 h at this temperature (Whyard et al., 1986). It is not known what the minimum duration, at or above this temperature, would be in order to induce synaptic thermoprotection. If synaptic thermoprotection could be induced within minutes after an initial heat stress, protection is not likely to act through the same mechanisms as acquired thermotolerance, nor could it involve upregulated HSPs acting directly in the synapse because time would be required for their synthesis and mobilization to sites of action (Tomasovic et al., 1985).

Unveiling the sites of action would be beneficial in attempting to understand the mechanisms involved in thermoprotection. Targeting specific areas and/or proteins for pharmacological intervention would bypass the need to use a heat shock, which can have wide ranging effects on all systems, to induce protection. Structures with which HSPs interact give an indication of where and how protection could be mediated. Various HSPs are known to interact with the cytoskeleton, and it is one of the earliest and most sensitive targets of stress (Dalle-Donne et al., 2001).

The specific goals of this study were: (1) to determine if failure temperature is time dependent by increasing the ramp rate of the stress test; (2) to determine if protection could be induced by an acute hyperthermic stress (minutes of exposure to temperatures above 45°C) by comparing failure temperatures of consecutive temperature ramps in the same preparation; (3) to examine the role of cellular structural elements in conferring and maintaining synaptic thermoprotection of synaptic transmission by pharmacologically altering cytoskeletal integrity with colchicine, which is a plant alkaloid that disrupts microtubule polymerization in the cytoplasm (Furcht and Scott, 1975), concanavalin A, which is a plant lectin that stabilizes microfilaments (McClain and Edelman, 1978), and cytochalasin B, which destabilizes microfilaments (Wheeler et al., 1985).

METHODS

Animals

All experiments were performed on the extensor tibiae (ETi) muscle of adult *Locusta migratoria* 2–5 weeks postimaginal molt. Animals were collected from a crowded colony. The colony was kept on a 16:8 hour light:dark cycle.

Preparation

The metathoracic ganglion and ETi muscle were exposed (Fig. 1) as described by Hoyle (1978). Nerve 5 was severed proximally and the axon of the fast extensor tibiae motoneuron was stimulated using suprathreshold square voltage pulses of duration 0.5 ms at 1 Hz. Responses were recorded from individual ETi muscle fibres using glass microelec-

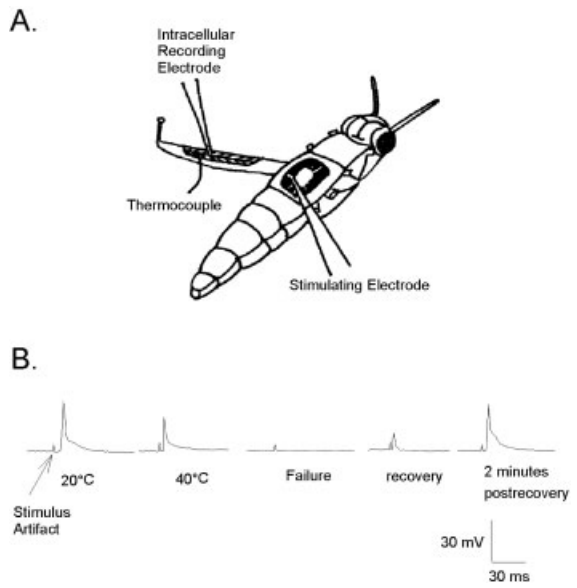

Figure 1 Locust extensor tibiae neuromuscular preparation. (A) Schematic of preparation (adapted from Barclay and Robertson, 2000). (B) Representative traces of EJPs during temperature ramp protocol in which saline was heated at a rate of 5°C/min. Turning the saline heater off results in a return of the saline to room temperature, ultimately leading to a recovery of muscular excitation.

trodes filled with 1 M KAc (resistance of 40–80 MΩ). Intracellular recordings were amplified using an Axoclamp-2A amplifier, and digitized using a Digidata 1200 (Axon Instruments, Foster City, CA). Axoscope data analysis software was used for analyzing excitatory junctional potential (EJP) amplitudes. Graph Pad Prism software was used for calculating area under the temperature versus time relationships (varying temperature ramp protocols).

Treatment

Heat-shocked animals were placed in a bucket in a humid oven for 3 h at 45°C, which induces a maximal heat-shock response in locusts (Whyard et al., 1986). At least 1 to 3 h was allowed for recovery following heat shock before the temperature ramp stress test was carried out. Control animals were kept in a similar bucket for 4 h at room temperature prior to dissection. Chemical treatment included exposure of the muscle to a 1-h bath application prior to the temperature ramp stress test. Cytochalasin B (cytoB) (1.0×10^{-5} *M*), concanavalin A (conA) (5.0×10^{-6} *M*) and colchicine (1.0×10^{-4} *M*) were added to the physiological saline described below. One-hour bath application of conA has been shown to induce significant effects on glutamate receptors in locust muscle (Mathers and Usherwood, 1978), and 1-h bath application of colchicine reveals significant disruption of microtubules in cockroach muscle (Furcht and Scott, 1975). All chemicals were purchased from Sigma-Aldrich.

Locust saline, at pH 7.2, composed of (in m*M*/L) 147 NaCl, 10 KCl, 4 $CaCl_2$, 3 NaOH, 10 HEPES was superfused over the muscle, and heated using a Nichrome heating coil wrapped around the glass perfusion pipette. Thermosensitivity of synaptic transmission was assessed by determining the operating limit of EJPs during a 5°/min temperature ramp (unless otherwise stated). The heater was turned off as soon as no EJP was discernible and room temperature was reattained within a few minutes. Subsequent ramps were undertaken only if amplitude had recovered to at least 1/4 the original amplitude within 5 min of recovery. Up to 5 additional minutes were allowed for EJPs to stabilize before subsequent ramps were undertaken. Saline temperature was monitored using a copper constantan thermocouple (0.2 mm diameter, Bat-12, Sensortek, Clifton, NJ) placed adjacent to the recording electrode. EJP amplitude decreased as temperature increased until no discernible response was visible [Fig. 1(B)]. Removal of the hyperthermic stress following EJP failure resulted in a subsequent recovery of the response.

Statistical comparisons were performed using SigmaStat software. Significant differences ($p < 0.05$) between treatments were assessed using unpaired *t*-test or nonparametric Mann-Whitney rank sum test.

RESULTS

Temporal Effects of Stress on Failure Temperature of Synaptic Transmission

The combined effects of stress intensity and duration of exposure are illustrated in Figure 2(A). Shown are the temperature recordings over time throughout the entire temperature ramp (each plot ends at failure), of five sequential ramps in one preparation. The fastest temperature ramp caused failure of synaptic transmission at the highest temperature, and the slower ramps failed at much lower temperatures.

We quantified the stress dose applied to the system by simply calculating the temperature multiplied by the time, or area under the temperature versus time graph. However, because time spent at temperatures within the normal operating range of the animal would clearly not contribute to the overall dose of stress applied to the system, it should not be included in the stress dose (area) measurement. Therefore a "threshold of stress" was determined, from which area under the graph, and above the threshold, was calculated. Area was calculated using several putative "stress thresholds" and the average of each of the five ramps and the standard deviation are shown in Table 1. Standard deviation was lowest at 34°C, suggesting this temperature to be the "threshold of stress."

Increasing the ramp rate to approximately 15°C/min resulted in an average failure temperature of 58.6

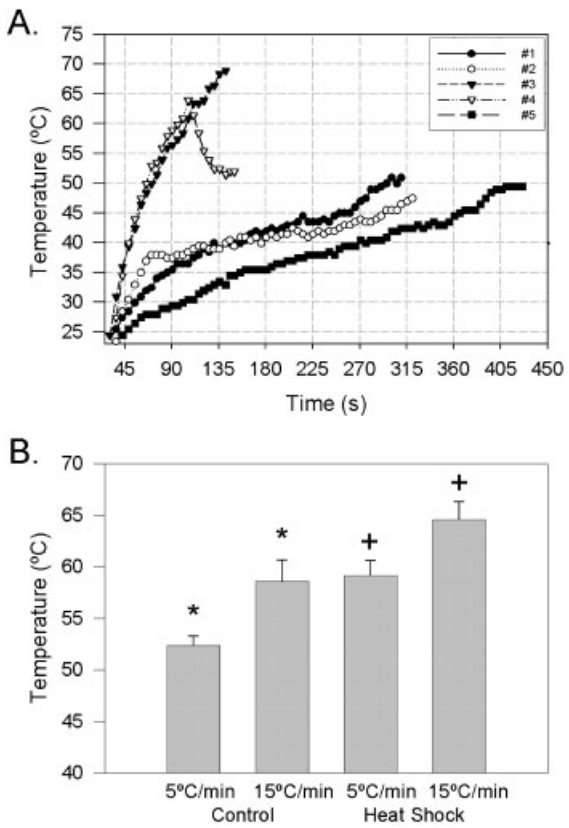

Figure 2 Temporal effects of stress exposure during temperature ramp. (A) Temperature (°C) recorded over time (s) during sequential, widely varying temperature ramp protocols, in one animal. Temperature recordings ended once neuromuscular transmission failed. (B) Two ramp speeds were used to compare effects of exposure duration on failure temperature. A faster temperature ramp of 15°C/min resulted in an elevation in failure temperature of 6.2°C in control animals and of 5.4°C in heat-shocked animals. (* and $^{+}$ statistical significance using *t*-tests, comparisons of failure temperature were made only within treatments) Values are means ± S.E.

± 2.1°C in controls, which is a significant increase of 6.2°C [*t*-test, $t(22) = 3.13$, $p = 0.005$]. A similar increase occurred in heat-shocked animals. Temperature at failure increased by 5.4°C to 64.6 ±1.7°C [*t*-test, $t(17) = 2.40$, $p = 0.031$] as a result of the increased ramp rate [Fig. 2(B)].

Failure Temperature of Synaptic Transmission after Acute Hyperthermic Stresses

The intensity of the hyperthermic stress exposure through each ramp surpasses the intensity used to induce synaptic thermoprotection in our heat-shock protocol (45°C). We therefore predicted that if there were acute effects of heat exposure on neuromuscular transmission it would be evident during a second temperature ramp.

Table 1 Area under the Temperature versus Time Graph [see Fig 3(A)] Was Calculated for Each of Five Subsequent Temperature Ramps Carried Out on One Preparation

Putative Threshold of Stress	Average Area (°C · s)	Standard Deviation
39°C	1107	454
36°C	1614	262
34°C	1975	122
31°C	2559	440
23°C	4892	1622

Because time spent at temperatures within the normal operating range of the locust would not contribute to the overall stress dose, several putative thresholds of stress were used to determine which would give the most accurate measurement. Standard deviation revealed the least difference between averages of the five experiments was at 34°C.

No acute effects of an initial temperature ramp were seen on failure temperature during subsequent ramps. Temperature-induced failure of synaptic transmission was not significantly different between first and second temperature ramps, even though the temperature of the initial ramp surpassed the temperature used in the heat shock protocol. Failure occurred at an average temperature of 52.4 ± 0.9°C during an initial ramp of 5°C/min and at 52.3 ± 1.4°C during the second ramp (*t*-test, $t(19) = 0.01$, $p = 0.967$] (Fig. 3).

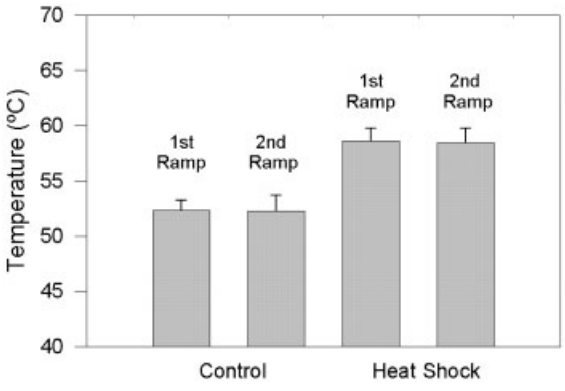

Figure 3 Acute hyperthermic stress does not alter failure temperature in short term. (A) Temperature at which neuromuscular transmission failed during a 5°C/min temperature ramp was assessed in control and heat-shocked animals. Following recovery of transmission a second temperature ramp was undertaken to assess any acute effects of the stress applied during the initial temperature ramp. Acute effects were not observed in control or heat shocked animals. Values are means ± S.E.

Table 2 Failure Temperatures in Sequential Heat Ramps (Ramp 1, Ramp 2, etc.) Normalized to the Failure Temperature of the First Heat Ramp and Measure in Eight Separate Preparations

Prep.	Ramp 1	Ramp 2	Ramp 3	Ramp 4	Ramp 5
1	1.00	0.99	0.96		
2	1.00	1.02	1.03		
3	1.00	1.01	1.02		
4	1.00	0.99	1.04		
5	1.00	1.05	1.03		
6	1.00	0.95	0.97	0.99	
7	1.00	0.97	0.98	1.00	0.99
8	1.00	1.03	1.05	1.02	1.02

In three preparations it was possible to deliver more than three ramps. There is no effect of multiple heat ramps on failure temperature.

Heat-shocked animals failed at 58.6 ± 1.2°C, indicating an increase of 6.3°C compared to controls [*t*-test, $t(25) = 4.20$, $p < 0.001$] (Fig. 2). This fits within the range of previously published results on heat-shock–induced thermoprotection of insect neural function (Dawson-Scully, 1998; Barclay and Robertson 2000, 2003). Failure temperatures in the second temperature ramps of heat-shocked animals were 58.4 ± 1.3°C and not different from the failure temperatures in the first temperature ramps [*t*-test, $t(23) = 0.11$, $p = 0.91$].

Results from eight experiments shown in Table 2 reveal that failure temperature fluctuated very little within animals across subsequent temperature ramps, even up to almost 1 h after the beginning of the initial stress. Recovered responses fail at the same temperature during subsequent temperature ramps.

Cytoskeletal Involvement in Synaptic Thermoprotection

To investigate the putative role of the cytoskeleton in conferring acquired thermoprotection, pharmacological manipulations were carried out. Application of 0.1 m*M* colchicine, a microtubule disrupter, did not have a significant effect [*t*-test, $t(20) = 0.67$, $P = 0.51$] on EJP failure temperature of heat shocked animals [Fig. 4(A)] that was 57.8 ± 1.7°C. However, time to recovery was significantly increased from 10.8 ± 3.0 s in heat-shocked animals to 74.4 ± 30.8 s in heat-shocked animals that had been treated with colchicine [*t*-test, $t(17) = 3.30$, $P = 0.0045$], and the latter were not different from controls [Fig. 4(B)]. This suggests that colchicine may interfere with the synapse's ability to restore itself. A second temperature ramp was undertaken in heat shocked animals exposed to colchicine, which showed a significant decrease in failure temperature of 4.6°C from 57.9 ± 1.7°C to 53.2 ± 0.9°C [*t*-test, $t(16) = 2.30$, $P = 0.034$] [Fig. 4(A)]. No significant effects of colchicine were observed on parameters of the EJP (data not shown).

ConA induced thermoprotection in a manner similar to heat shock. Heat shock increased the upper temperature limit of neuromuscular function from 51.3 to 59.7°C [*t*-test, $t(12) = 6.0$, $P = 0.001$) [Fig. 5(A)]. Synaptic recovery time after failure decreased significantly from 126.4 ± 47.9 s to 7.6 ± 3.1 s (Mann-Whitney U, $t = 29$, $p = 0.001$) [Fig. 5(B)]. Similarly, after conA exposure the upper temperature limit of synaptic transmission in the fast extensor

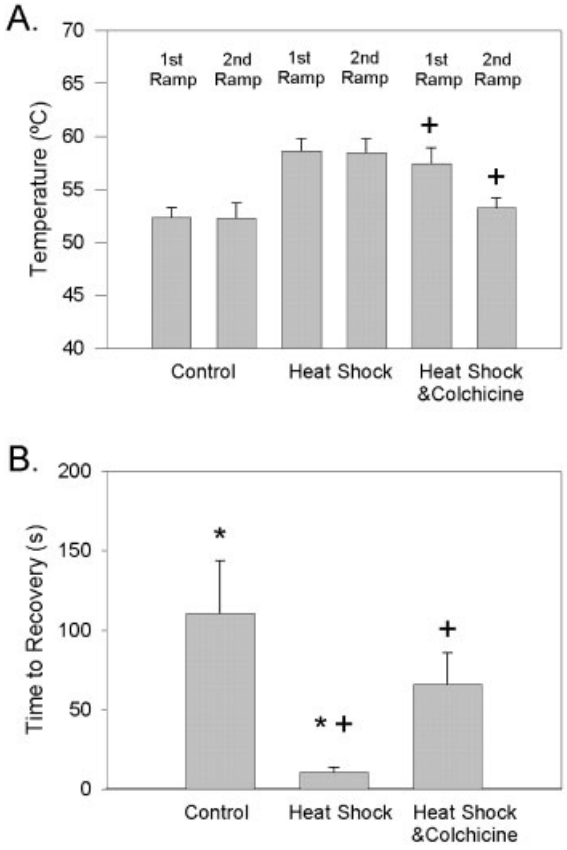

Figure 4 Effects of microtubule disrupter colchicine on failure temperature and recovery time. (A) Failure temperatures in heat-shocked animals that were exposed to colchicine were not significantly different from heat-shocked animals in the first temperature ramp. However, second temperature ramps revealed a significant decrease in failure temperature ($^{+}$indicates statistical significance using *t*-tests, comparisons of failure temperature were made only within treatments). (B) Recovery time in colchicine-exposed heat-shocked animals was significantly increased and not different from control animals (* and $^{+}$ statistical significance using *t*-test). Values are means ± S.E.

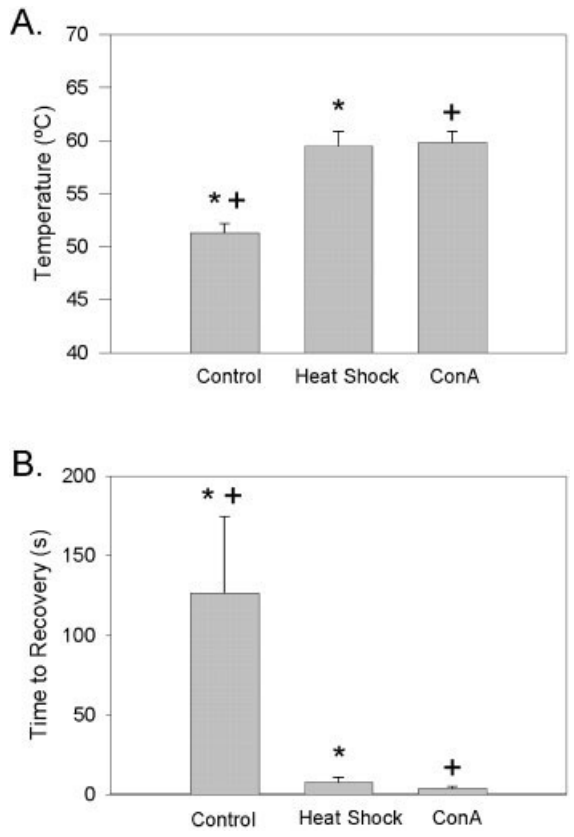

Figure 5 Concanavalin A mimics protective effects of heat shock on neuromuscular transmission compared to heat shock. (A) Failure limit of synaptic transmission was increased by application of 5.0×10^{-6} *M* conA. (B) Recovery time of synaptic transmission following failure was decreased dramatically by conA exposure. (* and $^{+}$ statistical significance using *t*-tests). Values are means ± S.E.

tibiae muscle was increased from 51.3 ± 0.9 to 59.8 ± 1.0°C [*t*-test, $t(12) = 6.3$, $P = 0.00004$) [Fig. 5(A)]. Synaptic recovery time after failure occurs decreased significantly from 126.4 ± 47.9 s to 3.8 ± 1.0 s [*t*-test, $t(15) = 2.7$, $p = 0.016$) [Fig. 5(B)] after conA exposure.

To determine if both conA and heat shock induced thermoprotection through cytoskeletal interactions of microfilaments, cytochalasin B, a microfilament disrupter was tested on both treatments. CytoB significantly decreased the failure temperature of conA protected animals from 59.83 ± 1.0°C to 55.0 ± 1.1°C [*t*-test, $t(12) = 3.25$, $p = 0.007$) [Fig. 6(A)] and increased the recovery times from 3.8 ± 1.0 s to 28.0 ± 10.0 s (Mann-Whitney U, $t = 56$, $p = 0.016$) [Fig. 6(B)]. CytoB diminished heat shock-induced thermoprotection [Fig. 6(B)] decreasing failure temperature by 6.2 to 53.3°C, which was comparable to control levels [*t*-test, $t(15) = 3.73$, $p = 0.002$]. Recovery time was increased from 7.6 ± 3.1 s to 70.0 ± 54.0 s, which was not different from controls [Fig. 6(D)]. Control animals exposed to cytoB showed no significant effect on failure temperature or recovery time.

DISCUSSION

Our goal is to understand how synaptic thermoprotection occurs and whether it is conferred through the same mechanisms as acquired thermotolerance. We characterized synaptic thermoprotection by examining temporal parameters of the acute stress response to the stress test itself. We also pharmacologically manipulated elements of the cytoskeleton to determine if its stability is related to the acquisition and maintenance of synaptic thermoprotection. This study shows that heat-shock–induced protection of synaptic transmission is not fast acting, and that failure temperature is dependent on both the duration and the intensity of the hyperthermic stress applied to the system. We also demonstrate that elements of the cytoskeleton are involved in conferring and maintaining synaptic thermoprotection.

Stress Dose

Altering the ramp rate altered the failure temperature. This was not surprising since an abundance of literature exists on the combined effects of stress intensity and the duration of exposure (Gerner, 1987). Two ramp rates, 5°C/min and 15°C/min were used to determine if failure temperature was time-dependent. Synaptic transmission failed 6°C higher using the faster ramp rate. In cardiac cells the temperature ramp rate significantly affects the rate of HSP synthesis and the degree of acquired thermotolerance (Tomasovic et al., 1985). Our assessment of "stress dose" illustrates two fundamental points. First, a threshold exists above which, the effects of stress begin to accumulate ultimately leading to failure of synaptic transmission. Second, the higher the stress intensity the faster the "stress dose" accumulates and the sooner failure occurs. The threshold temperature for inducing acquired thermotolerance in our colony is 37°C (Whyard et al., 1986). The threshold of stress was calculated to be between 34 and 36°C.

Lack of Acute Hyperthermic Stress Effect on Failure Temperature

Synaptic thermoprotection was not induced in the short term. Saline temperature at failure of transmission in all experiments surpasses the temperature used

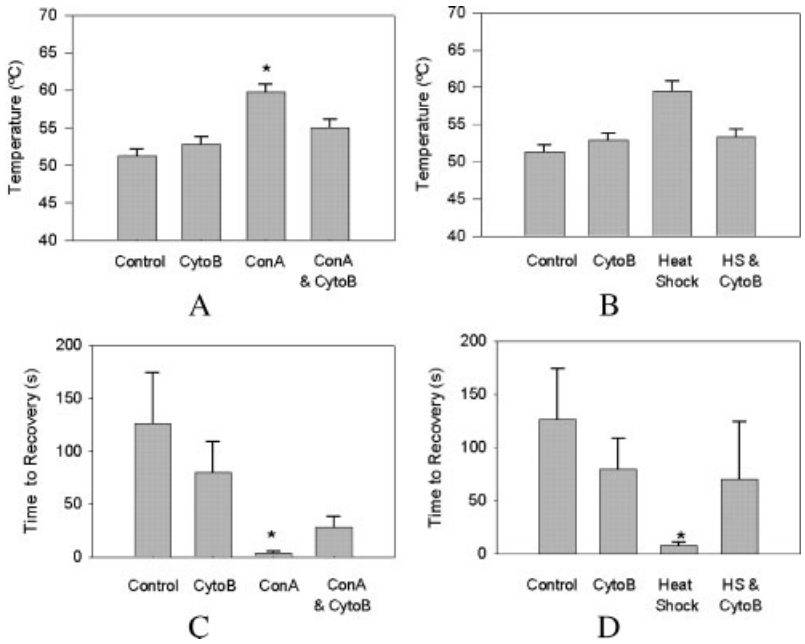

Figure 6 Concanvalin A-induced and heat-shock–induced thermoprotection of neuromuscular transmission is disrupted by cytochalasin B Cytochalasin B disrupted both (A) conA-mediated and (B) heat shock-mediated elevation of failure temperature. Cytochalasin B also disrupted both (C) conA–mediated and (D) heat shock-mediated reduction in recovery time (* statistical significance from each of the other test groups using *t*-tests). Values are means ± S.E.

in our heat-shock protocol (45°); however, failure temperature is not altered in subsequent ramps. Data presented here reveal that there was no acute effect or fast acting component to synaptic thermoprotection, even after numerous temperature ramps continuing almost an hour after the initial temperature ramp. This parallels the delay between a heat shock and the appearance of both upregulated heat-shock proteins and acquired thermotolerance (Tomasovic et al., 1985; Whyard et al., 1986). In *Drosophila* it has been shown that synaptic thermoprotection diminishes 6 h after heat shock, when upregulated HSPs are no longer detectable (Karunanithi et al., 1999). Taken together, these data are consistent with a role for upregulated heat-shock proteins in conferring synaptic thermoprotection.

Also, heat-shocked animals were still protected during second ramps. This reveals that protective mechanisms are long lasting and are not compromised during exposure to extreme temperatures reached at the point of transmission failure. The temperature of the saline in these experiments can surpass 60°C, intensities at which many proteins would begin to denature. HSPs, however, are known to operate at temperatures higher than most constitutively produced proteins and it is believed that a trigger of HSP synthesis may involve proteins that have denatured from an initial stress (Sharp et al., 1999). This may further support a role for HSP involvement in synaptic thermoprotection.

Heat Shock and the Cytoskeleton

Substantial evidence exists supporting a role for upregulated HSPs in conferring acquired thermotolerance (Parsell et al., 1993). The structures HSPs interact with give an indication of where and how protection could be mediated. The cytoskeleton is one of the earliest and most sensitive cellular targets of stress and numerous HSPs interact with it (Dalle-Donne et al., 2001). Hsp 90 and hsp 70 interact with microtubules while the smaller HSPs namely hsp 27 and α-crystallin interact with microfilaments (Liang and MacRae, 1997).

The cytoskeleton can contribute to activity-dependent processes underlying synaptic plasticity through regulation of cellular morphology and signaling proteins. Functions of the cytoskeleton include sculpting cell morphology, exo- and endocytosis, cell division, cell polarity, intracellular migration, intercellular adhesion, signal transduction, and regulation of ion channel populations and activity (Molitoris, 1997). Stress can cause microtubules to disassemble, intermediate filaments to "cave in" towards the nucleus,

and actin microfilament integrity to disrupt resulting in dissociation from the cell membrane (Molitoris et al., 1997; Loktionova et al., 1999; Dalle-Donne et al., 2001).

Heat-shock proteins protect cytoskeletal integrity during stress, likely mediating, at least partially, acquired thermotolerance. α-Crystallin can prevent heat-induced aggregation of actin filaments by stabilizing actin polymers (Wang and Spector, 1996). Overexpression of hsp 27 after a heat shock results in increased microfilament stability, accelerated recovery, and increased survival rate of cells (Lavoie et al., 1993a, 1993b; Huot et al., 1995).

The plant alkaloid colchicine, which binds to tubulin dimers and disrupts their polymerization into microtubules (Furcht and Scott, 1975), was used to examine the effect of microtubule disruption on synaptic thermoprotection. Although there was no significant decrease in failure temperature of animals exposed to colchicine in the first ramp, a significant increase in time to recovery occurred and a decrease in failure temperature of second ramps was shown. Perhaps the effects of colchicine are not seen in the initial ramps because the site of action is not accessed until failure in the first ramp occurs. However, similar concentrations show significant disruption of microtubules after 1 h in cockroach neurons (Kuster et al., 1983). Because colchicine acts by preventing tubulin from polymerizing into microtubules (Furcht and Scott, 1975) perhaps during the first ramp temperature-induced tubulin disruption occurs and is then prevented from satisfactorily repolymerizing, resulting in the synapse's lost ability to maintain protection. Colchicine, which binds to isolated membrane fractions (Wunderlich et al., 1973), is known to reversibly alter the interaction of tubulin with cell membranes (Becker et al., 1975).

ConA was shown to induce synaptic thermoprotection paralleling effects of a heat shock. It has many physiological effects including polymerizing actin (Rao and Varani, 1982), and stabilizing cell shape (Lin and Huestis, 1995). ConA has been shown to alter the mobility of various cell surface receptors and it has been suggested that anchoring of conA receptors results from cytoskeletal interactions (McClain and Edelman, 1978; Jung et al., 1984). In addition, conA reduces glutamate receptor desensitization in locust (Mathers and Usherwood, 1978) and crayfish muscle (Klose et al., 2002). Desensitization is the reduction of a response due to a repeated or long-lasting stimulus, a phenomenon that can be temperature-sensitive (Salanki et al., 1989). Desensitization of receptors occurs within milliseconds (Sun et al., 2002) and is therefore not likely to be involved in the time-dependent process leading to failure.

The cytoskeleton has been shown to alter active properties of the membrane as well, including receptor kinetics. Calcium-dependent inactivation of NMDA receptors is known to involve α-actinin, which links the receptor to actin filaments (Krupp et al., 1999). In our study the EJP duration at half-maximal amplitude is not altered by heat-shock or conA exposure (data not shown) suggesting channel kinetics are not altered.

Microfilament disruption by cytochalasin B interferes with conA and heat-shock–induced synaptic thermoprotection. This suggest a possible common mechanism involving structural stabilization of the cytoskeleton. Maintenance of the integrity of the postsynaptic density relies on structures in the subsynaptic reticulum such as members of the MAGUK family of proteins. These proteins are responsible for anchoring signaling proteins, such as glutamate receptors, through binding to elements of the cytoskeleton (Sheng and Pak, 1999). Long-term facilitation and long-term depression have both been correlated with alterations in glutamate receptor density (Carroll et al., 1999), and various glutamate receptors have been shown to redistribute away from active zones within as little as 5 min at room temperature (Carroll et al., 1999; Lissin et al., 1999). Glutamate receptors are known not only to internalize but also to move relatively large distances laterally in the membrane (Borgdorff and Choquet, 2002). Localization and mobility of glutamate receptors can be manipulated through cytoskeletal disruption (Allison et al., 1998, 2000). Presynaptic targets must be considered as possible sites of action as well. Actin has been implicated in mechanisms that maintain vesicles within reserve pools as well as in their translocation to the presynaptic membrane (Doussau and Augustine, 2000). Many actin-binding proteins are regulated by calcium, suggesting mechanisms may go beyond vesicle tethering and movement (Doussau and Augustine, 2000).

Synaptically located microtubules and microfilaments appear to be involved in conferring and maintaining acquired thermotolerance. Experiments in this article suggest that synaptic thermoprotection, like acquired thermotolerance, may be acting through mechanisms involving interactions between the cytoskeleton and heat shock proteins. This robust preparation's ability to withstand repeated and long duration stresses is ideal for the above experiments. However, further experiments utilizing targeted genetic manipulations would aid in more specifically determining which proteins are interacting with the cytoskeleton to confer synaptic thermoprotection.

This preparation is not amenable to genetic manipulation suggesting *Drosophila* as a future consideration for use as a model preparation.

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