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Comparative Biochemistry and Physiology, Part A 147 (2007) 344–348
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Cytoskeletal stability and heat shock-mediated thermoprotection of central pattern generation in *Locusta migratoria*

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Received 14 July 2006; received in revised form 30 October 2006; accepted 31 October 2006
Available online 20 February 2007

Abstract

Prior exposure to extreme temperatures can induce thermoprotection in migratory locusts, which is important for survival in their natural environment. An important motor activity that needs to be protected is ventilation. The mechanism underlying heat shock is not fully understood, and our goal was to test the idea that cytoskeletal stability is critical for such thermoprotection. Cytoskeletal stabilizers (concanavalin A) and destabilizers (colchicine) were bath-applied in semi-intact locust preparations in both control (C) and pre-treated heat-shocked (3 h, 45 °C) animals. We measured parameters of the ventilatory motor pattern during maintained high temperature (43 °C) and recorded the times taken for motor pattern generation to fail and then recover on returning to room temperature. We found that concanavalin A mimicked the effects of a prior heat stress in control animals by increasing time to failure and decreasing time to recovery of motor pattern generation. However, colchicine destroyed protection in heat-shocked animals by decreasing time to failure and increasing time to recovery. Our findings confirm that the cytoskeleton has a mechanistic role in preserving neural function at high temperatures, possibly through stabilizing ion channels and other integral membrane proteins (e.g. Na^+/K^+ ATPase) and their interactions with heat shock proteins.

Keywords: Colchicine; Concanavalin A; Cytoskeleton; Locusts; Thermoprotection

1. Introduction

Many different organisms are exposed to stress in one form or another, which can lead to impairments of nervous system function and eventually death. In this study we were primarily concerned with the deleterious effects of high temperatures on neural function in the model system, *Locusta migratoria*. In their natural environment, these locusts are exposed to a wide range of temperatures, and being ectotherms, are extremely susceptible to changes in ambient temperature. However, it has been found that physiological protective mechanisms have evolved to cope with environmental stress, and these differ from behavioural responses such as avoidance that are not sufficient to combat extremely high temperatures (Robertson et al., 1996; Robertson, 2004). In addition, the neural circuits vital to survival during episodes of high temperature stress are experimentally accessible, allowing comprehensive studies at the cellular level in live and semi-intact preparations.

Previous studies have shown that protection of organisms from environmental stress can be induced in the short term through prior exposures to stress. It has been found that previous oxidative stress can lead to adaptations (e.g. antioxidant defence and expression of heat-shock proteins) that protect organisms from subsequent stress (Dalle-Donne et al., 2001). In terms of thermoprotection, a high, sub-lethal temperature exposure (e.g. heat shock) can induce tolerance in organisms to a subsequent exposure of high temperatures (Whyard et al., 1986). Locusts incubated at 45 °C for 3 h (Heat Shocked, HS) are found to display thermoprotection of neural function in subsequent exposure to high temperatures.

A crucial motor activity that must be protected during periods of high temperature stress is ventilation in order to maintain circulation of respiratory gases. Ventilatory motor pattern generation is controlled by a neural circuit known as a central pattern generator (CPG), located within the metathoracic ganglion of

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doi:10.1016/j.cbpa.2006.10.044

locusts (Bustami and Hustert, 2000). The CPG is a network of partly known interneurons feeding into motor neurons that then activate ventilatory muscles to contract (Ramirez and Pearson, 1989; Bustami and Hustert, 2000). Research has shown this ventilatory network is sensitive to various kinds of stress such as extreme temperatures (Robertson, 2004) and prior heat stress leads to thermotolerance and thermoprotection of ventilatory motor pattern generation (Newman et al., 2003). Thus, ventilation is a good motor activity to study the underlying physiological effects of the heat shock response on neural function, which remain only partly understood.

The mechanisms underlying thermoprotection of neural function in locusts remain to be determined. Heat shock proteins (Hsps) have been implicated in playing diverse roles in cell functions of many organisms and act as molecular chaperones to minimize undesirable protein folding and interactions which may occur because of exposure to high temperatures (Feder and Hofmann, 1999), thus allowing locusts to cope with this environmental stress (Whyard et al., 1986; Qin et al., 2003). One element in the cell that Hsps interact with is the cytoskeleton (Dalle-Donne et al., 2001), making it an interesting avenue to investigate. The properties and organization of the cytoskeleton play a large role in maintaining cell morphology, vesicle movement, cell division, and signal transduction, and are thus essential to proper cell function (Richter-Landsberg and Goldbaum, 2003). The cytoskeleton is also known to interact with the membrane, allowing stabilization of important membrane processes (Dai and Sheetz, 1999). Any disruption in the cytoskeleton can lead to adverse cellular effects, and it has been found that the cytoskeleton is one of the earliest and most sensitive targets of stress (Dalle-Donne et al., 2001). Prior heat stress has been found to stabilize the actin cytoskeleton; however the mechanism behind this stabilization remains unknown, although various heat shock proteins are believed to mediate this protection (Wang et al., 2001). To investigate a potential role of the cytoskeleton in conferring thermoprotection of the CPG underlying ventilation in locusts, cytoskeletal stabilizers and destabilizers were bath-applied to semi-intact locust preparations. Colchicine has many known biological effects, including the binding to, and disassembly of microtubules through the prevention of tubulin polymerization (Sato et al., 1988). Concanavalin A acts on actin filaments of the cytoskeleton, and is known to induce the polymerization of actin in rats (Rao and Varani, 1982), as well as induce synaptic thermoprotection in locust neuromuscular junctions, thus mimicking the effects of a prior heat shock in control locusts (Klose et al., 2004).

2. Materials and methods

2.1. Animals

Adult male locusts, *L. migratoria*, 2–4 weeks after imaginal ecdysis, were collected from a crowded colony in the animal care facility at Queen's University. Locusts in the colony were exposed to a photoperiod of 12 h of light and 12 h of darkness, and maintained at a temperature of 25 °C.

2.2. Experimental pre-treatment

Locusts were distributed among 6 experimental groups: control (C; $n=10$), heat shock (HS; $n=9$), control/colchicine (C/Col; $n=10$), control/concanavalin A (C/ConA; $n=10$), heat shock/colchicine (HS/Col; $n=10$) and heat shock/concanavalin A (HS/ConA; $n=10$). Control animals were collected and placed in a ventilated 2 L plastic container for 4 h at room temperature. C/ConA and C/Col groups received the same pre-treatment. Locusts receiving heat shock pre-treatment were placed in an incubator at 45 °C for 3 h, followed by 1 h of recovery after incubation. HS/Col and HS/ConA received same pre-treatment as HS animals.

2.3. Experimental preparation

A live, semi-intact locust set-up was used to expose the ventilatory muscles within the abdomen (Newman et al., 2003). After removing legs, wings, and pronotum, a dorsal incision was made down the midline to open the thorax, and the locust was pinned onto a cork surface dorsal side up. All fat bodies, air sacs, and the gut were removed, while nerves and muscle 161 remained intact. Dissections were performed under standard locust saline containing (mM/L): 147 NaCl, 10 KCl, 4 $CaCl_2$, 3 NaOH, and 10 Hepes buffer (pH 7.2). A Peri-Star peristaltic pump (World Precision Instruments Inc., Sarasota, FL, USA) was used for saline inflow for 20 min following dissection. Saline flowed onto the metathoracic ganglion to ensure temperature manipulation of the ventilatory CPG, and continued to flow posteriorly through the animal. Saline was heated using a Nichrome heating coil wrapped around a glass perfusion pipette, and controlled by varying the current passing through the wire. A thermocouple (Bat-12, Physitemp Instruments, Clifton, NJ, USA) placed near the metathoracic ganglion was used to monitor changes in temperature. For drug treatments, colchicine (1.0×10^{-4} M; Sigma-Aldrich, St. Louis, MO, USA) and conA (5.0×10^{-6} M; Sigma) were added to the standard locust saline described above, and allowed to flow over the preparation for 20 min prior to the temperature ramp. These concentrations were shown to be effective in destabilizing and stabilizing cytoskeletal elements, respectively, at the neuromuscular junction in locusts (Klose et al., 2004).

2.4. Extracellular recording

Ventilatory motor patterns were recorded from muscle 161 using an electromyographic electrode (EMG) comprised of a 0.1 mm diameter copper wire insulated except at the tip. Ventilatory rhythms were displayed using Axoscope 9.0 (Axon Instruments Inc., Union City, CA, USA), and allowed to stabilize for 20 min before temperature increase. A temperature ramp increased saline temperature in the locust at a rate of 5 °C/min from room temperature to 43 °C. At 43 °C, the temperature was held for 30 min or until failure of the ventilatory motor pattern. Animals failing at a temperature below 43 °C were characterized to have a time to failure of zero seconds. Time to failure, (characterized by the time to a loss of motor pattern generation

once at 43 °C) was recorded, and temperature was allowed to return to room temperature for 30 min, or until recovery. Time to recovery was described as the time following heat-induced failure until the first sign of a ventilatory rhythm. Animals that did not recover were characterized to have a recovery time of 30 min. Mean times to failure and recovery were plotted using Sigma Plot 9.0, and compared across the treatment groups using *t*-tests and Mann–Whitney Rank Sum tests using Sigma Stat 3.0. Standard error of the means (SEM) was also plotted for each treatment group.

3. Results

3.1. Control and heat-shocked pre-treatments

Locusts previously incubated for 3 h at 45 °C were found to display thermotolerance when compared to controls. Half of control animals failed at temperatures less than 43 °C during temperature ramps, compared with only 10% of heat-shocked animals. 100% of all animals in the control and heat-shocked groups recovered, and time to failure and time to recovery were compared between control and heat-shocked locusts. A significantly longer time to failure was found in heat-shocked animals compared with control animals (Mann–Whitney Rank Sum Test, $p=0.008$) (Fig. 1A). There was no significant difference in time to recovery found between controls and heat-shocked animals (Mann–Whitney Rank Sum Test, $p=0.241$) (Fig. 1B).

3.2. Influence of cytoskeletal elements on time to failure

Colchicine and concanavalin A (conA) were bath-applied to test for an influence of the cytoskeleton in conferring thermoprotection. Heat-shocked locusts bath-applied with colchicine took a significantly shorter time to fail than heat-shocked locusts (Mann–Whitney Rank Sum Test, $p=0.025$) (Fig. 1A). However, bath application of conA to heat-shocked locusts had no observable effect on time to failure when compared to heat-shocked locusts (Fig. 1A). There was a significant difference between controls bathed in conA and control animals, with the conA-control locusts having a longer time to failure (similar to heat-shocked animals) than controls not treated with conA (Mann–Whitney Sum Rank Test, $p=0.006$) (Fig. 1A). Bath application of colchicine to controls had no observable effect on time to failure compared to control locusts with no colchicine treatment (Fig. 1A).

3.3. Influence of cytoskeletal elements on time to recovery

Time to recovery of the ventilatory motor pattern was also measured after treatment with colchicine and conA. Heat-shocked locusts bath-applied with colchicine demonstrated a significantly longer time to recovery than heat-shocked animals with no colchicine application (*t*-test, $t=-2.572$, $df=18$, $p=0.019$) (Fig. 1B). Heat-shocked animals bathed in conA displayed no observable difference from the standard heat-shocked animals (Fig. 1B). There was a significant difference between control animals bathed with conA and the standard control animals, with control/conA locusts displaying a shorter time to recovery, similar to heat-shocked animals (Mann−Whitney Rank Sum Test, $p=0.026$) (Fig. 1B). Controls bathed in colchicine displayed no observable differences compared with the non-colchicine treated control group (Fig. 1B).

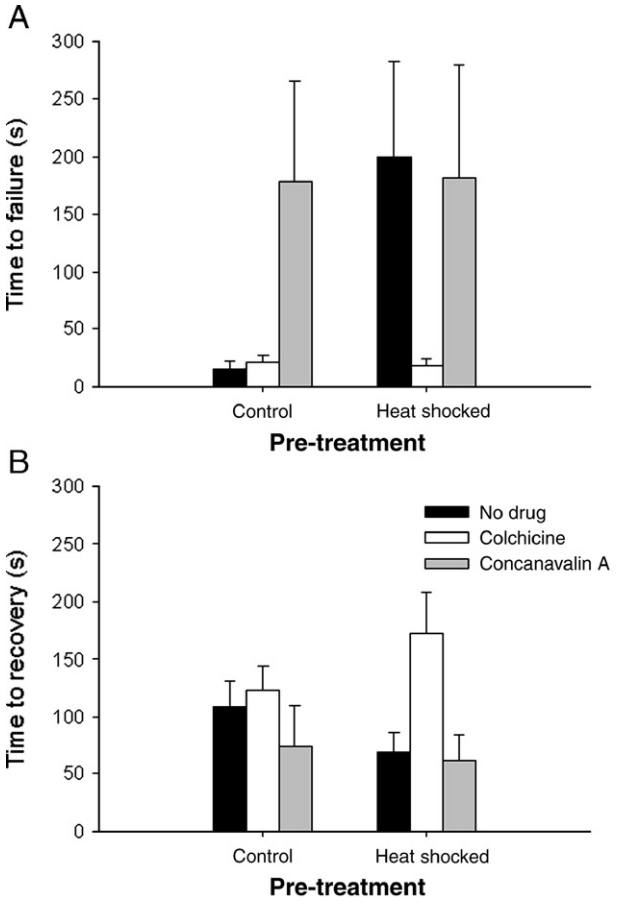

Fig. 1. Times to failure and recovery of ventilatory motor pattern generation across different treatment groups. A. Time to failure of motor pattern generation in control ($n=10$), heat-shocked ($n=9$), colchicine-treated (1.0×10^{-4} M; $n=10$ for each pre-treatment), and conA-treated (5.0×10^{-6} M; $n=10$ for each pre-treatment) locust preparations. B. Time to recovery of motor pattern generation following heat-induced failure in control, heat-shocked, colchicine, and conA-treated groups. Error bars represent standard error of the mean for each treatment group (refer to Results for statistical details).

4. Discussion

Neural control of ventilatory motor pattern generation by the CPG is sensitive to high temperatures, and locusts have developed mechanisms to combat this stress. Exposure to high, sublethal temperatures can induce thermotolerance upon subsequent exposures, and this heat shock response has been found within the CPG controlling ventilatory motor patterns (Newman et al., 2003). The mechanisms underlying the induction of thermotolerance in response to a previous heat stress remain to be found, however this study examined possible roles of the cytoskeleton in conferring thermoprotection of a neural circuit. It is possible the cytoskeleton plays an intermediary role in the

heat shock response in the ventilatory CPG, but it was first necessary to show that the integrity of cytoskeletal elements is involved in conferring thermoprotection.

4.1. Control vs. heat-shocked locusts

Time to failure differed between control and heat-shocked locusts, and ventilatory motor pattern generation failed significantly faster in control than pre-stressed locusts, as found in previous studies (Newman et al., 2003). There was no significant difference in time to recovery between control and heat-shocked animals, which differs from the study by Newman et al. (2003). In their study, Newman et al. measured time to recovery as the time recorded from heat-induced failure to first sign of movement in the expiratory muscles, whereas our study measured time to recovery as first detection of a ventilatory rhythm after heat-induced failure. This difference in methodology could account for differences in the measurement of time to recovery, as well as any potential significant differences between pre-treatment groups.

A possible explanation for increased time to failure and decreased time to recovery in heat-shocked locusts is the enhancement of short-term synaptic plasticity in pre-stressed locusts. It has been found that a heat shock pre-treatment (3 h/45 °C) increases neural activity and modifies synapse performance in locusts (Barclay and Robertson, 2001). This might explain our results that heat-shocked animals displayed longer times to failure and shorter times to recovery.

4.2. Cytoskeletal involvement in thermoprotection of neural function

Colchicine was found to decrease time to failure and increase time to recovery in heat-shocked locusts, thus disrupting the heat-shock induced thermoprotection of neural function. Concanavalin A (conA), a microfilament stabilizer, exhibited the opposite effects of colchicine in controls. The cytoskeleton is involved in many cellular functions. Cytoskeletal elements (e.g. microtubules and actin) are involved in cell morphology, as well as multiple membrane interactions such as endocytosis, exocytosis, cell division, signal transduction, and ion channel activity and regulation (Molitoris, 1997). When exposed to stress, actin filaments are known to disorganize/sever, and microtubules disassemble (Dalle-Donne et al., 2001; Mounier and Arrigo, 2002).

These effects of stress on the cytoskeleton are important in maintaining proper neural function. At high temperatures, the lipid bilayer of neural cell membranes can dissociate, changing membrane potentials, and lead to many changes in neural function including spontaneous activity of action potentials (APs) or loss of proteins critical for maintaining the integrity of the bilayer (Luna and Hitt, 1992, Wu et al., 2001). This is important because the cytoskeleton has major interactions with the cell membrane and its associated proteins (Luna and Hitt, 1992), and damage to the cytoskeleton or proteins it associates with may lead to changes in neural function. Observations of colchicine-sensitive proteins such as microtubules suggest an influence of this drug on the distribution of intrinsic membrane proteins (Furcht and Scott, 1975).

In neurons, the actin cytoskeleton is involved in functions including growth and synaptic function, and along with its associations with the plasma membrane is believed to play a large role in ion channel function (Prat and Cantiello, 1996; Maguire et al., 1998). Disruption of the actin cytoskeleton by cytochalasin D (a microfilament destabilizer) reduced NMDA channel activity in rats (Rosenmund and Westbrook, 1993), and other changes in the actin cytoskeleton can influence voltage-gated K^+ activity, and the regulation of other ion channels in neurons (Maguire et al., 1998). Application of colchicine depresses synaptic transmission in *Aplysia californica* ganglion cell synapses through the depression of Ca^{2+} currents (Baux et al., 1981) and inhibits mechanosensitive ion channel activity (Su et al., 2000) implicating microtubules having important roles in ion channel function.

In locusts, a heat shock pre-treatment has long-term effects on K^+ currents, and Hsps may be directly or indirectly involved in the modification of potassium channels leading to these effects (Ramirez et al., 1999). Hsps interact with the cytoskeleton, suggesting an intermediary role of the cytoskeleton in this study. Findings that ion channel activity in neurons is regulated by actin and microtubules suggest a large influence of cytoskeletal elements on neural function (Prat and Cantiello, 1996).

Heat shock proteins interact with both microtubules and microfilaments. Large Hsps, including Hsp90 and Hsp70 bind to the microtubule network (Mounier and Arrigo, 2002), likely regulating microtubule assembly/disassembly (Liang and MacRae, 1997). Small Hsps interact with actin filaments playing a role in maintaining their integrity and guarding them from heat shock effects (Liang and MacRae, 1997). A model has been proposed for how small Hsps interact with, and protect the actin cytoskeleton. In this model, small Hsps may form large non-phosphorylated aggregates in non-stressed cells; however, upon a heat stress, the first response is a phosphorylation of small Hsps and disruption of these large aggregates (Mounier and Arrigo, 2002). These small clusters of small Hsps would interact directly with actin, or actin associated proteins, to protect actin filaments from destabilizing and promote their reorganization (Mounier and Arrigo, 2002).

This study has shown that the cytoskeleton is involved in the heat shock response; however the exact mechanisms of how the cytoskeleton is involved remain unclear. Our results suggest a testable hypothesis on the cytoskeleton's role in preserving neural function at high temperature, which might depend upon their interactions with Hsps and other integral membrane proteins. A within animal study measuring potassium channel activity using TEA and the action of cytoskeletal drugs may yield important clues in the future into the mechanism of the heat shock response in locusts.

Acknowledgements

This work was supported by an NSERC grant, and we would like to thank Corinne I. Rodgers for editorial comments on a

previous version of the manuscript, as well as technical assistance with the experiments.

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