Anoxia induces thermotolerance in the locust flight system

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Summary

Heat shock and anoxia are environmental stresses that are known to trigger similar cellular responses. In this study, we used the locust to examine stress cross-tolerance by investigating the consequences of a prior anoxic stress on the effects of a subsequent high-temperature stress. Anoxic stress and heat shock induced thermotolerance by increasing the ability of intact locusts to survive normally lethal temperatures. To determine whether induced thermotolerance observed in the intact animal was correlated with electrophysiological changes, we measured whole-cell K^+ currents and action potentials from locust neurons. K^+ currents recorded from thoracic neuron somata were reduced after anoxic stress and decreased with increases in temperature. Prior anoxic stress and heat shock increased the upper temperature limit for generation of an action potential during a subsequent heat stress. Although anoxia induced thermotolerance in the locust flight system, a prior heat shock did not protect locusts from a subsequent anoxic stress. To determine whether changes in bioenergetic status were implicated in whole-animal cross-tolerance, phosphagen levels and rates of mitochondrial respiration were assayed. Heat shock alone had no effect on bioenergetic status. Prior heat shock allowed rapid recovery after normally lethal heat stress but afforded no protection after a subsequent anoxic stress. Heat shock also afforded no protection against disruption of bioenergetic status after a subsequent exercise stress. These metabolite studies are consistent with the electrophysiological data that demonstrate that a prior exposure to anoxia can have protective effects against high-temperature stress but that heat shock does not induce tolerance to anoxia.

Key words: anoxia, thermotolerance, locust, *Locusta migratoria*, flight, K^+ current.

Introduction

Many organisms are challenged by exposure to low-oxygen and high-temperature stress in their natural environment. Anoxic and high-temperature stress activate similar cellular stress pathways (Morimoto et al., 1992), disrupt the bioenergetic state of neurons and consequently compromise cellular health and neural circuit function. A cellular response activated by both anoxic and high-temperature stress is the rapid production of a family of highly conserved heat-shock proteins (hsps). This heat-shock response is a universal response to stress that leads to cytoprotection from a variety of subsequent stressors including hypoxia/ischaemia (Morimoto and Santoro, 1998; Sharp et al., 1999). However, it is less clear whether such cross-tolerance results in equal measures of protection against different stresses at the level of the whole organism, particularly whether hypoxia pre-treatment can mitigate the effects of a subsequent heat stress. We have investigated the interactions between effects of prior anoxia and prior heat shock in the African migratory locust *Locusta migratoria*, an organism that is very tolerant to both low-oxygen and high-temperature stress.

The specific effects of anoxic stress are organism- and tissue-dependent. In neurons and muscle, effects may include excitotoxicity and bioenergetic disturbance. Anoxic stress in hypoxia-sensitive vertebrates is associated with increases in extracellular excitatory amino acids leading to increased Ca^{2+} influx (Silver and Erecinska, 1990; Szatkowski and Attwell, 1994). High levels of intracellular Ca^{2+} then trigger a cascade of events leading to neuronal damage or death (Choi, 1995; Tymianski et al., 1994). Changes in neuronal membrane potential are also observed after oxygen deprivation and are associated with modulation of K^+ currents (Dool et al., 1991; Nieber et al., 1995; Leblond and Krnjevic, 1989; Trapp and Ballanyi, 1995). In hypoxia-tolerant vertebrates such as turtles and fish, a switch to anaerobic metabolism compensates for a lack of oxygen and ion channel activity is down-regulated during anoxia, decreasing the demand for ATP (Hochachka et al., 1996; Bickler and Buck, 1998). Evidence for channel modulation and arrest has been found in a variety of channels including those for Na^+, Ca^{2+} and K^+ (Hochachka et al., 1996; Bickler and Buck, 1998; Perez-Pinzon et al., 1992; Pek and Lutz, 1997). Insects also survive oxygen deprivation but have evolved different survival strategies from those found in hypoxia-tolerant vertebrates. Cockroaches (*Periplaneta*

americana) are able to survive several hours under hypoxic conditions. Despite a rapid depletion of ATP and consequent paralysis, these insects recover fully upon returning to normoxic conditions (Pitman, 1988). Like cockroaches, fruit flies (*Drosophila melanogaster*) are extremely tolerant to oxygen deprivation, and exposure to hypoxia can alter neuronal excitability and signalling [cockroach (Pitman, 1988; Le Corronc et al., 1999); fruit fly (Gu and Haddad, 1999; Haddad, 2000)].

In locusts, heat shock increases the survival rate of animals subsequently exposed to the normally lethal temperature of 53 °C (Whyard et al., 1986;, Robertson et al., 1996). Prior heat shock can also exert neuroprotective effects that can be extended to electrophysiological properties of neurons associated with induced thermotolerance of signalling events (Dawson-Scully and Robertson, 1998; Karunanithi et al., 1999; Ramirez et al., 1999; Robertson et al., 1996). Heat shock depresses neuronal K^+ efflux by causing rapid inactivation of currents (Ramirez et al., 1999). During an energetic stress, decreases in whole-cell outward K^+ currents in locust neurons probably serve the same purpose as the downregulation of channel activity in turtles; it would minimize demand on ATP consumption by decreasing the amount of K^+ that would need to be actively pumped back into the cell to restore ionic concentration gradients across the membrane (Ramirez et al., 1999; Pek and Lutz, 1997). Heat shock has also been shown to be neuroprotective by increasing the upper temperature limit for action potential generation (Wu et al., 2001), synaptic transmission (Dawson-Scully and Robertson, 1998), neuromuscular transmission (Barclay and Robertson, 2000) and the generation of motor output (Robertson et al., 1996) during a subsequent heat stress. In other model systems, heat shock protects against a variety of conditions including apoptosis, excitotoxicity and oxygen deprivation (Lowenstein et al., 1991; Mailhos et al., 1993; Amin et al., 1995; Morimoto and Santoro, 1998; Ikeda et al., 2000).

Such protective effects of brief hyperthermia can be mimicked by hypoxia treatment. Specifically, prior exposure to either hyperthermia or hypoxia increases the survival rate of retinal ganglion cells subsequently exposed to anoxia and excitotoxicity (Caprioli et al., 1996). Although there is ample evidence demonstrating the protective effects of heat shock, little is known about the long-term effects of anoxia on the cross-tolerance of electrical signalling. Characterizing mechanisms involved in cross-tolerance of electrical signalling can lead to an understanding of important pathways that modulate neuronal circuitry such that adaptive behaviours may be preserved during stress.

In this study, stress cross-tolerance in the locust flight system was examined at the whole-animal, neuronal and muscular levels using a variety of electrophysiological and biochemical approaches. These investigations have allowed us to conclude that, whereas similarities may exist in response to different stressors at the cellular level, this may not be reflected in the responses of the organism as a whole.

Materials and methods

Animals and experimental treatments

Male *Locusta migratoria* L., at least 2 weeks past imaginal ecdysis, were collected from a crowded colony. The colony was maintained at 30 °C on a 16 h:8 h light: dark cycle as described previously (Robertson et al., 1996). Locusts receiving heat-shock (HS) treatment were placed in a 2 l container and kept for 3 h at 45 °C in a humid oven. Control animals were kept under similar conditions at room temperature (23±1 °C). Locusts receiving anoxia treatment were exposed to an atmosphere of pure nitrogen for 1–6 h depending on the experiment. Experiments were performed 1–5 h after heat shock or anoxic treatment. Exercised locusts were obtained by flying control and heat-shocked locusts for 45 min in a stream of air. Each insect was tethered at the pronotum with wax to a copper rod and suspended in a stream of wind created by room fans for 45 min at 25 °C. Locusts that stopped flying were tapped with a stick to induce flying again. Locusts that did not fly or repeatedly stopped flying such that they did not fly for the entire time period were excluded from the experiment.

Thermotolerance test

Forty-five locusts were distributed equally among three experimental groups: control, heat-shock and anoxic. Heat-shock locusts were heat-shocked and placed at room temperature for 1 h before the thermotolerance test. Anoxic locusts were exposed to nitrogen for 2 h and then placed under normoxic conditions at room temperature for 1 h before the thermotolerance test. Animals were labelled on their wing using a felt-tip marker to indicate their experimental group. After the 1 h recovery period, all locusts were placed into a single 2 l container that was subsequently placed into a humid oven at 53 °C. Locusts that were assumed to be dead were removed at 30 min intervals. All removed animals were kept at room temperature for 24 h to ensure that they were dead.

Tolerance to anoxic stress

Tolerance to anoxic conditions was assessed in control and heat-shock locusts (1 h post treatment). Locusts were placed in a 2 l glass container at room temperature that was subsequently flooded with nitrogen. At 1 h intervals up to 6 h, seven locusts from each experimental population (42 control and 42 heat-shock) were removed and placed under normoxic conditions at 25 °C. Time to recover was scored as the time required to 'self-right' (i.e. stand upright).

Electrophysiology

Patch-clamp recording

To expose and isolate the metathoracic ganglion for patch-clamp experiments, a dorsal midline incision of the thorax was made. Locusts were then decapitated and the gut was removed. The animals were dissected in standard extracellular saline containing (in mmol l^{-1}): 128 NaCl, 24 NaHCO$_3$, 1 NaH$_2$PO$_4$, 1 MgSO$_4$, 3 KCl, 30 D-glucose, 1 CaCl$_2$, equilibrated with continuous bubbling of carbogen (95 % O$_2$/5 % CO$_2$) to a pH

of 7.4 (310 osmol kg^{-1}). All the metathoracic nerves as well as the mesothoracic connectives leading to the metathoracic ganglion were severed. The isolated ganglion was then attached with a small amount of cyanoacrylate glue to an agar block with the ventral side facing outwards and the rostral side oriented upwards. The tissue and agar block were secured in a tissue slicer, submerged in a bath of ice-cold saline and aerated continuously with carbogen. Transverse slices were made from the rostral to the caudal end of the ganglion to expose a section approximately 200 μm into the ganglion. From this rostral boundary, a 400 μm slice was made and immediately transported to a tissue chamber where it was stabilized with a handmade titanium harp, strung with nylon thread. The tissue was submerged in a constant flow-through of carbogen-aerated saline with an initial temperature of 25 °C and allowed to stabilize under these conditions for 10 min prior to recording. Slices of both control and anoxia-pre-treated animals were used for up to 1 h following the start of animal dissection.

Whole-cell patch-clamp recordings were obtained using unpolished glass patch pipettes. Pipettes had an open tip resistance of 2–4 MΩ when filled with an internal pipette solution containing (in mmol l^{-1}): 5 NaCl, 140 KCl, 1 MgCl$_2$, 10 EGTA, 4 Na$_2$ATP, 0.2 CaCl$_2$ and 10 Hepes corrected to a pH of 7.2 using KOH. Recordings were obtained with a visual patch-clamp technique using a patch pipette under positive pressure positioned with a PCS-5000 micromanipulator (Burleigh Instruments, Inc.). Junction potentials were nulled prior to seal formation. Release of pressure and a gentle suction resulted in a cell-attached patch with a gigaohm seal. After rupture of the cell membrane with additional suction, a whole-cell configuration was achieved, and recordings were made at 5 °C increments starting at 25 °C. Cell size was determined using both the estimated cell diameter, as it appeared on the calibrated television monitor, and the whole-cell capacitance, as read on the Axopatch amplifier. Only cells with diameters between 20 and 40 μm were selected for experiments.

Whole-cell voltage-clamp recordings were acquired using an Axopatch-1D amplifier (Axon Instruments, Inc.), and programs for controlling and analyzing patch-clamp experiments were created using pCLAMP 6 software in conjunction with a Digidata 1200 interface (Axon Instruments, Inc.). Evoked currents were recorded with leak subtraction (monitored constantly) either on- or off-line. Currents were evoked by depolarizing voltage steps from –80 to 40 mV from holding potentials (V_h) of either –60 mV or –40 mV. Voltage steps were made in 10 mV increments with a duration of 200 ms.

Only recordings obtained from neurons with an initial seal resistance of 2 GΩ were included in the analysis. All currents included in the analysis and in representative traces are from recordings made with leak subtraction off-line. Manual leak subtraction was performed using pCLAMP 6 software (Axon Instruments, Inc.) to calculate linear leak subtraction at each voltage step and then subtracted to determine the leak current from the total current. Currents were filtered at 2 kHz and digitized at 10 kHz. Series resistance was typically 1.24 MΩ

and was compensated by 80 %. Cell capacitance was compensated using the nulling circuitry of the recording amplifier. Current traces were filtered using a 200 Hz low-pass filter before performing the analysis.

For some patch-clamp experiments, K$^+$ currents were blocked with application of tetraethylamonium chloride (TEA) saline containing (in mmol l^{-1}): 103 NaCl, 24 NaHCO$_3$, 1 NaH$_2$PO$_4$, 1 MgSO$_4$, 3 KCl, 30 D-glucose, 1.5 CaCl$_2$, 25 TEA with an osmolality of 310 osmol kg^{-1} and equilibrated to a pH of 7.4 using NaOH. TEA was added to the saline just prior to use in experiments.

Intracellular recording

Locusts were heat-shocked or exposed to anoxic conditions for 2 h (as outlined above) and allowed to recover for 1 h at room temperature before experiments were performed. The animals were pinned ventral side down and dissected to expose the ventral nerve cord. The meso- and metathoracic ganglia were then removed and placed in saline containing (in mmol l^{-1}): 147 NaCl, 10 KCl, 4 CaCl$_2$, 3 NaOH and 10 Hepes and saturated with carbogen. The ganglia were pinned ventral side down on a silicone elastomer (Sylgard) and bathed with the saline.

Action potentials were evoked with a stimulating electrode on nerve 3 of the mesothoracic ganglion and were recorded intracellularly with glass microelectrodes (1 mol l^{-1} potassium acetate, 40 MΩ) from the neuropil segments of forewing motoneurons (Fig. 1). The temperature of the saline was controlled using a heating coil of Nichrome wire wrapped around the inlet pipette and was monitored with a thermocouple (Bat-12, Physitemp Instruments Inc.) adjacent to the mesothoracic ganglion. The temperature of the saline flow bathing the ganglia was increased until an action potential could no longer be generated (i.e. failure). The saline was then allowed to cool to 25 °C to allow recovery. Recovery was monitored and scored as the time required to generate a just noticeable action potential after heat-induced failure, provided that the penetration remained stable after heat-induced failure. Signals were amplified (Getting, model 5), recorded to videotape *via* Neuro-Corder (DR 890) and subsequently digitized and analyzed (Digidata 1200, Axoscope, Axon Instruments). Action potentials were characterized by measuring time to peak, latency, duration at half-amplitude and amplitude (see Fig. 1). If the stimulus artefact overlapped substantially with the action potentials, these recordings were included only in the failure temperature and recovery data.

Respiration of isolated mitochondria

Mitochondrial isolation

Muscle mitochondria were isolated as described by Suarez and Moyes (1992), as adapted from Chappell and Hansford (1972). Locusts were decapitated, and the abdomen, appendages and thoracic fat bodies were removed. Muscle from 4–6 locusts was dissected for each mitochondrial preparation. All further isolation processing was carried out on ice in isolation buffer consisting of (in mmol l^{-1}): 250 sucrose,
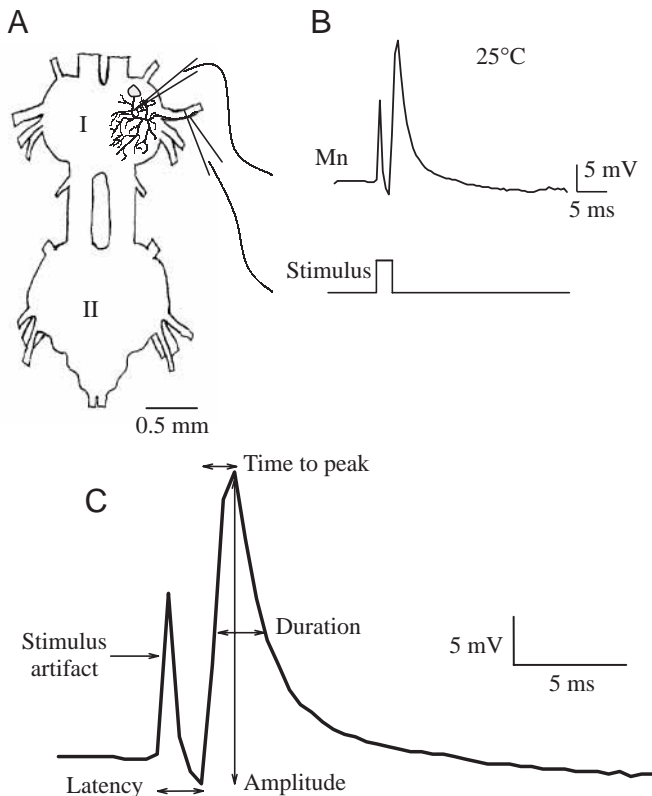
Fig. 1. Diagram of an isolated locust thoracic ganglia preparation for intracellular recording from motoneurons. (A) The meso- (I) and meta- (II) thoracic ganglia with a flight muscle motoneuron. In this preparation, the motoneuron is stimulated extracellularly and recorded from intracellularly from the neuropil. (B) Sample trace of an action potential recorded from a motoneuron (Mn) at 25 °C. Note the stimulus artefact preceding the action potential. (C) Sample trace of an action potential trace showing the parameters that were measured. Note the stimulus artefact preceding the action potential.

1 EGTA and 10 Tris-HCl (pH 7.4 at 25 °C). Protease (Neutralase: ICN) at 15 mg per 30 ml total volume was added, and flight muscle was homogenised by three low-speed passes in a Potter-Elvejhem homogeniser with a loose-fitting Teflon pestle. After 10 min, the homogenate was diluted with 30 ml of isolation medium containing 1 % fatty-acid-free bovine serum albumin (BSA isolation buffer), poured through eight layers of cheesecloth into a chilled beaker and centrifuged (3 min, $3000\,g$). The resulting supernatant was centrifuged (3 min, $7500\,g$) to obtain the mitochondrial pellet. Any partially digested myofibrils on top of the pellet were removed with each centrifugation. The pellet was resuspended in BSA isolation buffer and recentrifuged (5 min, $7500\,g$). The pellet was resuspended, recentrifuged and resuspended in isolation medium to a final concentration of approximately $10–15\,\mathrm{mg\,ml^{-1}}$.

Substrate oxidation rates

Assays were conducted at 25 °C in 2.2 ml of assay medium in a water-jacketed cell with a removable injection port maintaining temperature with a Lauda circulating water bath. Oxygen consumption rates were measured polarographically using a Clarke-type oxygen electrode interfaced with Vernier Instruments Data Logger software. The mitochondrial suspension (100 µl) was added to the assay buffer consisting of (in $\mathrm{mmol\,l^{-1}}$): 154 KCl, 1 EGTA, 25 KH_2PO_4, 1 % BSA and 10 Tris-HCl (pH 7.4 at 25 °C). Mitochondrial respiration was measured after the addition of pyruvate ($1\,\mathrm{mmol\,l^{-1}}$) and malate ($0.25\,\mathrm{mmol\,l^{-1}}$). ADP ($0.25\,\mathrm{mmol\,l^{-1}}$) was added to determine state III respiration, which represents O_2 consumption during active synthesis of ATP. After all the ADP had been phosphorylated, respiration returned to the state IV rate. The respiratory control index (RCI) was determined by dividing state III respiration by state IV respiration. Trials in which the RCI was below 10 were excluded from analyses. Mitochondrial oxygen consumption was expressed as $\mathrm{nmol\,O_2\,min^{-1}\,unit^{-1}}$ citrate synthase activity. The activity of citrate synthase (CS) was determined with mitochondrial extracts solubilized in an equal volume of $20\,\mathrm{mmol\,l^{-1}}$ Hepes (pH 7.4), $1\,\mathrm{mmol\,l^{-1}}$ EDTA and 0.1 % Triton X-100. Enzyme activities were assayed in microplates (Molecular Devices Spectramax 200) at 25 °C in the presence of $50\,\mathrm{mmol\,l^{-1}}$ Tris-HCl (pH 8.0), $0.1\,\mathrm{mmol\,l^{-1}}$ 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), $0.12\,\mathrm{mmol\,l^{-1}}$ acetyl CoA and $0.5\,\mathrm{mmol\,l^{-1}}$ oxaloacetate (omitted for controls). CS activities per milligram mitochondrial protein in both heat-shock and control animals was $2.66\pm0.23\,\mathrm{units\,mg^{-1}}$ mitochondrial protein.

Metabolite analyses

Bioenergetic status was evaluated by measuring arginine phosphate (ArgP) and arginine (Arg) levels after heat shock and in relation to anoxia and recovery from anoxia. One index of energetic state is the 'arginine charge', which is the proportion of the total arginine pool that exists as ArgP: {[ArgP]/([ArgP]+[Arg])}. ArgP is an invertebrate energy reserve analogous to phosphocreatine of vertebrate muscle, replenishing or buffering ATP levels within the muscle during periods of high energy demand. This relationship between [ArgP] and [Arg] is pH-dependent (Wegener, 1996). During exercise, small decreases in [ATP] can occur despite large increases in ATP turnover; such energetic changes are often more easily seen as larger decreases in [ArgP] (Wegener, 1996). These limitations are minimized when samples are assayed in parallel on individuals and expressed as the ratio [ArgP]/([ArgP]+[Arg]).

Locusts were heat-shocked and subsequently exposed to anoxia 1 h later, as described previously. After anoxic treatment, control and heat-shock locusts were divided into 'no recovery' (animals that were dissected immediately after anoxic treatment) and 'recovery' (dissected after recovery determined by self-righting behaviour) groups. Thus, four experimental groups were studied: control, control with post-anoxia recovery, heat shock, heat shock with post-anoxia recovery.

Locusts used in metabolite analyses were decapitated, the abdomen and legs were removed and the thorax was rapidly

frozen in liquid nitrogen and stored at –80 °C. Whole thoraces were powdered in a mortar and pestle cooled in liquid nitrogen, then homogenized in 10 vols of 6 % perchloric acid (PCA). The homogenate was centrifuged (7 min at $5000\,g$), the supernatant neutralized (0.1 vol of saturated Tris base, 0.1 vol of $2\,mol\,l^{-1}$ KCl and 0.45 vol of $1\,mol\,l^{-1}$ KOH) and centrifuged (5 min at $10\,000\,g$). The pH of the final supernatant was adjusted to pH 7.0 (if necessary) with $1\,mol\,l^{-1}$ KOH, and the supernatant was stored at –80 °C. Fat body and flight muscle were dissected from control locust thoraces to estimate total phosphagen pools in these tissues.

Metabolites were analysed using standard NADH-linked enzymatic assays (Lowry and Passonneau, 1972). Assays were run in triplicate using $250\,\mu l$ of homogenate. Levels of ArgP were determined using glucose-6-phosphate dehydrogenase (G6PDH)-linked and hexokinase (HK)-linked assays. Samples $(20\,\mu l)$ were diluted with $80\,\mu l$ of water and mixed with $200\,\mu l$ of a solution containing $20\,mmol\,l^{-1}$ Tris-HCl (pH 8.0), 5 mmol l^{-1} glucose, $2\,mmol\,l^{-1}$ NAD^+, $5\,mmol\,l^{-1}$ $MgCl_2$, 0.1 units of G6PDH, 0.1 units of HK and 0.5 units of arginine kinase (ArgK). Controls lacked ArgK. Levels of arginine were determined by measuring the disappearance of NADH in the presence of pyruvate kinase (PK), lactate dehydrogenase (LDH) and ArgK. Samples $(50\,\mu l)$ were mixed with $50\,\mu l$ of water and $200\,\mu l$ of solution containing $50\,mmol\,l^{-1}$ Hepes (pH 7.0), $0.5\,mmol\,l^{-1}$ phosphoenolpyruvate, $0.25\,mmol\,l^{-1}$ NADH, $5\,mmol\,l^{-1}$ $MgCl_2$, 1.25 units of LDH, 1.25 units of PK and 0.5 units of ArgK. Controls lacked ArgK (for arginine).

Post-exercise metabolite analysis

Before determining the interactive effects of heat shock and exercise, we first reassessed the effects of heat shock alone on metabolic status (as described above). The bioenergetic consequences of recovery from heat shock and the interactive effects of heat shock and exercise were assessed 1 h after heat shock. Metabolite levels were then compared with those of control animals that had been left at room temperature for 4 h. The interactive effects of heat shock and exercise were measured after locusts had had a 1 h recovery period after heat shock.

Statistical analyses

Data were plotted using SigmaPlot 4.0 graphing software (Jandel Scientific, San Rafael, CA, USA). Quantitative data are presented as means ± standard error (S.E.M.). Statistical significance was assessed using either a *t*-test or a two-way analysis of variance (ANOVA), as indicated in the text. Statistical analysis was performed using SigmaStat 2.0 software (Jandel Scientific, San Rafael, CA, USA). Differences between means were considered significant when $P<0.05$.

Results
Thermotolerance test

Prior heat-shock (45 °C for 3 h) and anoxic (2 h in nitrogen) treatments increased the thermotolerance of locusts (Fig. 2).
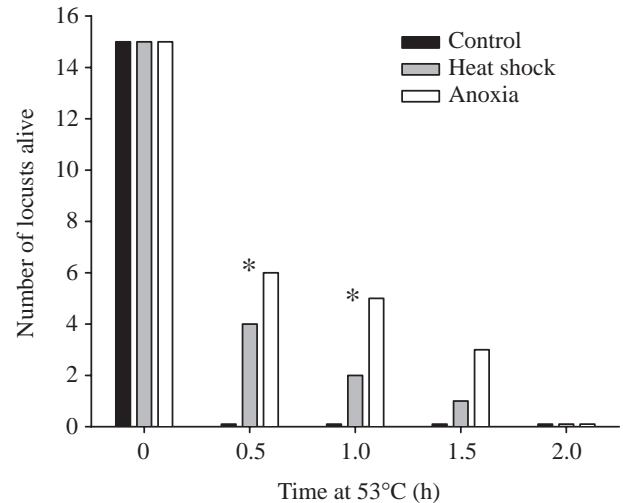

Fig. 2. The effect of prior heat shock (3 h at 45 °C) and anoxia (2 h in nitrogen) treatment on the survival of locusts at the normally lethal temperature of 53 °C. Heat shock and anoxia pre-treatment significantly altered the thermotolerance of locusts by increasing the number of locusts alive after 0.5 h (*G*-test, $G=10.09$, d.f.=2, $P<0.05$) and 1 h (*G*-test, $G=8.02$, d.f.=2, $P<0.05$) at 53 °C. Asterisks indicate a significant difference between control and experimental groups $(P<0.05)$.

After 0.5 h and 1 h at 53 °C, locusts that had been pre-treated with heat shock or anoxia had significantly higher survival rates than controls (Fig. 2) (0.5 h, *G*-test, $G=10.09$, d.f.=2, $P<0.05$; 1 h, *G*-test, $G=8.02$, d.f.=2, $P<0.05$), demonstrating that, at the level of whole-animal survival, cross-tolerance exists between an anoxic stress and a high-temperature stress.

K^+ currents

The whole-cell capacitance of neuronal somata selected for patch-clamp experiments did not differ between control $(1.96\pm0.40\,pF)$ and anoxia-treated $(2.57\pm0.36\,pF)$ animals (*t*-test, $t=1.40$, $P=0.27$, d.f.=15). Outward currents described in this study were identified as K^+ currents on the basis of the current/voltage relationships and activation curves and confirmed by application of TEA $(25\,mmol\,l^{-1})$, which blocked the outward currents (data not shown).

The relationships between evoked peak currents (*I*) and applied voltage steps (*V*) when $V_h=-60\,mV$ were determined for cells of control and anoxia-treated animals at 25 °C (Fig. 3A,B). Prior anoxia treatment significantly altered the mean *I/V* relationships of peak currents at a saline temperature of 25 °C (two-way ANOVA with Tukey pairwise multiple-comparison test, $F=2.98$, $P<0.001$, d.f.=12) (Fig. 3A,B).

To investigate the consequences of prior anoxia treatment on whole-cell conductance, activation curves for neurons of control and anoxia-treated animals were calculated. The activation curves were derived from peak currents recorded in response to the same voltage protocol at 25 °C. The whole-cell chord conductance was calculated for each voltage step as $G=I/(V-E_K)$, where *G* is the whole-cell conductance, *I* is the current amplitude

Fig. 3. Effects of prior exposure to anoxic conditions on whole-cell K$^+$ currents in locust neurons. Outward K$^+$ currents obtained with voltage steps made from a holding potential of –60 mV in 10 mV increments from –80 mV to 40 mV. (A) Representative whole-cell voltage-clamp recordings from neurons in locust metathoracic ganglion slices of a control and an anoxia-treated animal at 25 °C. (B) Mean I/V plots of control (N=9) and anoxia-treated (N=10) neurons at 25 °C. Prior anoxia treatment significantly affected the I/V relationship (two-way ANOVA with Tukey pairwise multiple-comparison test, F=2.98, d.f.=12, P<0.001). Following anoxia, less current was evoked with each voltage step compared with control recordings. Values are means ± S.E.M. (C) Mean activation curves of whole-cell K$^+$ currents recorded from control and anoxia-treated neurons at 25 °C. G is the whole-cell conductance; G_{max} is the maximal conductance at a

voltage step to 40 mV. $V_{1/2}$, the test potential at which there is half-maximal conductance, of control cells was –10.55±1.27 mV and did not differ from the $V_{1/2}$ of –7.27±2.01 mV for anoxia-treated cells (t-test, t=1.34, P=0.20, d.f.=17). Asterisks indicate a significant difference between control and experimental groups (P<0.05).
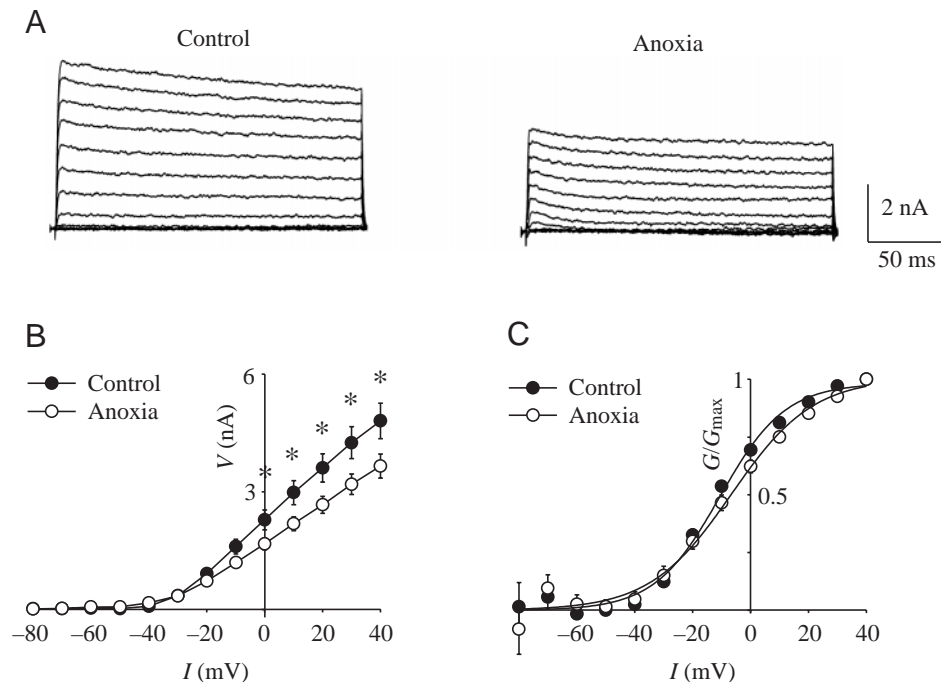
at each voltage step, V is the voltage step and E_K is assumed to be the Nernst K$^+$ equilibrium potential of –84 mV. Once the conductance at each potential had been determined, G/G_{max} was calculated. Activation curves were fitted to a Boltzmann equation, $G(V_m)=G_{max}/\{1+\exp[(V–V_{1/2})/K]\}$, using non-linear least-square fits, where $G(V_m)$ is the conductance at each voltage step, G_{max} is the maximal conductance at a voltage step to 40 mV, $V_{1/2}$ is the test potential at which conductance is half-maximal and K is the slope of the activation curve. G_{max} of control cells was 33.94±3.11 nS (N=9), a value different from the G_{max} of 26.05±2.12 nS found in anoxic cells (N=10) (t-test, t=2.13, P<0.05, d.f.=17). $V_{1/2}$ values of cells of anoxia-treated animals were not significantly different from those of cells of control animals (t-test, t=1.34, P=0.20, d.f.=17). Control $V_{1/2}$ for activation was –10.55±1.27 mV (N=9) compared with –7.27±2.01 mV (N=10) for cells of anoxia-treated animals (Fig. 3C).

The amount of current evoked decreased with increasing temperature, and this decrease was evident for both control and anoxia-treated neurons (Fig. 4A). Decreases in current with increasing temperature were reversible since current amplitude increased to its original value in experiments that returned saline temperature to 25 °C after an increase to 30 °C (data not shown). The temperature-sensitivity of these currents was also investigated. There was a significant effect of temperature on peak current amplitude (two-way ANOVA with Tukey pairwise multiple-comparison test, F=25.77, P<0.001, d.f.=2). Post-hoc analysis indicates that both control and anoxia-treated currents decreased significantly when the temperature was increased from 25 to 30 °C but not from 30 to 35 °C. There was

no significant interaction between anoxia treatment and temperature (two-way ANOVA with Tukey pairwise multiple-comparison test, F=0.75, P=0.48, d.f.=2).

There was a significant effect of anoxia treatment on peak current amplitude (two-way ANOVA with Tukey pairwise multiple-comparison test, F=4.58, P=0.04, d.f.=1). Peak currents of anoxia-treated animals were reduced compared with those of controls at 25 °C (Fig. 4B). However, there was no difference in peak current amplitude between control and anoxia-treated animals at 30 or 35 °C.

When peak currents at 30 and 35 °C were normalized to the evoked peak currents at 25 °C, control and anoxia peak currents changed in a similar fashion when the temperature was increased to 30 °C (t-test, t=0.41, P=0.69, d.f.=10) and then to 35 °C (t-test, t=0.18, P=0.86, d.f.=9) (Fig. 4C). However, in control slices, there was a significant difference between the normalized peak current at 30 °C and the normalized peak current at 35 °C (t-test, t=3.09, P=0.01, d.f.=9). This difference was not seen for the anoxia-treated animals (t-test, t=0.86, P=0.44, d.f.=10).

Action potentials

Intracellular recording from locust motoneurons in an isolated thoracic ganglia preparation revealed that heat-shock and anoxia pre-treatment increased the upper temperature limit for generation of an action potential (one-way ANOVA with Tukey pairwise multiple comparison, F=12.00, d.f.=2, P<0.001) from 36.9±0.6 °C (control, N=8) to 43.7±1.2 °C in heat shock (N=8) and 41.5±1.2 °C in anoxia (N=9). There was no difference in failure temperature between heat-shock- and

Fig. 4. The effect of prior anoxia treatment on whole-cell outward K+ currents of neurons in locust metathoracic ganglion slices with increasing temperature. Outward currents were obtained using voltage steps from a holding potential (V_h) of –60 mV made in 10 mV increments from –80 mV to 40 mV. (A) Representative traces of whole-cell outward K+ current recorded from neurons in locust metathoracic ganglion slices at different temperatures (25, 30 and 35 °C) showing the current amplitude for both control and anoxia-treated animals. (B) Peak currents after anoxia treatment. When the temperature was held at 25 °C, peak currents from neurons of control animals ($N=9$) were significantly greater than peak currents from cells of anoxia-treated animals ($N=10$) when $V_h=-60$ mV (two-way ANOVA with Tukey pairwise multiple-comparison test, $F=4.58$, $P=0.04$, d.f.=1). Anoxia treatment did not significantly alter the peak current evoked when $V_h=-60$ mV at 30 °C (control, $N=6$; anoxia, $N=6$) or at 35 °C (control, $N=5$; anoxia, $N=6$). Values are means + S.E.M. (C) Histograms of peak currents normalized to peak currents at 25 °C.
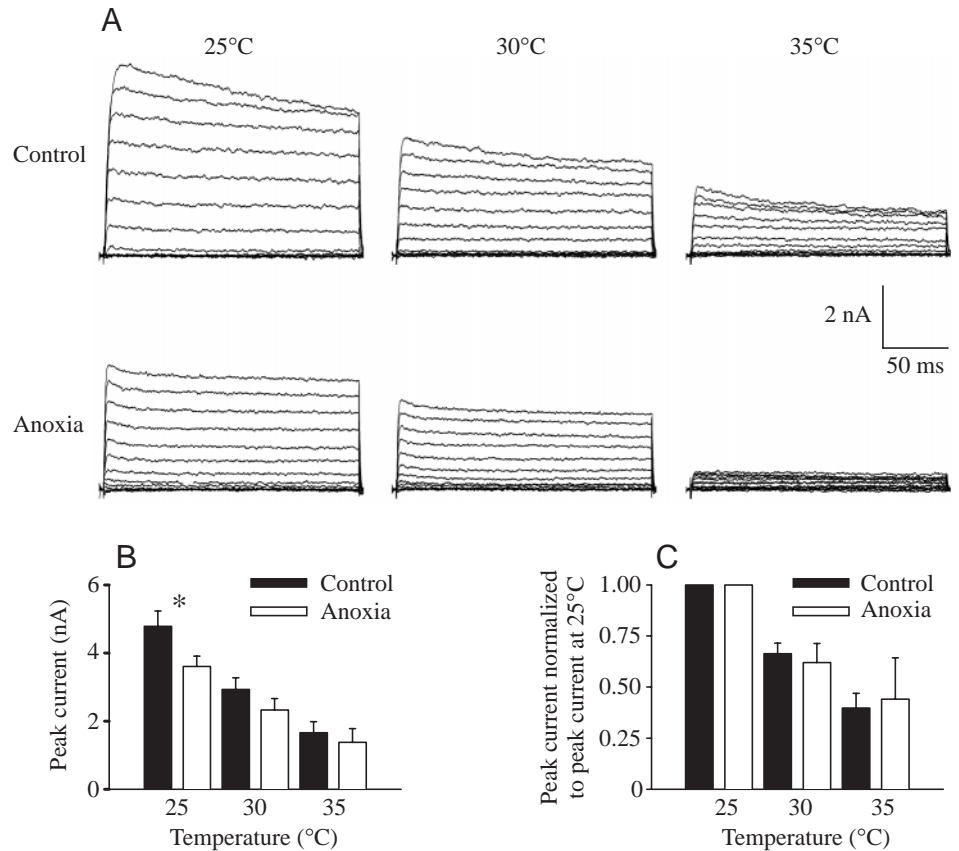
Control and anoxia currents were equally reduced when the temperature was increased from 25 to 30 °C and to 35 °C. Asterisks indicate a significant difference between control and experimental groups ($P<0.05$).

anoxia-treated neurons (one-way ANOVA, $F=1.20$, d.f.=2, $P=0.3$). There appeared to be a tendency for action potentials in heat-shock- (25±4.05 s, $N=5$) and anoxia-treated (27.4±11.7 s, $N=5$) neurons to recover more quickly than those in controls (66±23.28 s, $N=4$) after heat-induced failure; however, after analysis, there was no statistical difference in the time to recover (one-way ANOVA, $F=2.60$, d.f.=2, $P=0.12$). It was not possible to record time to recover for 11 neurons as a result of loss of penetration.

Heat shock and anoxia both altered the shape of motoneuron action potentials recorded intracellularly (Fig. 5). Increases in temperature decreased latency, time to peak, duration and amplitude in control, heat-shock and anoxia neurons (Fig. 5). Heat shock decreased the latency (Fig. 5A; two-way ANOVA with Tukey multiple pairwise comparisons, $F=6.74$, d.f.=2, $P<0.05$) and time to peak (Fig. 5B; two-way ANOVA with Tukey multiple pairwise comparisons, $F=7.02$, d.f.=2, $P<0.05$) of action potentials compared with both control and anoxic neurons. Anoxia increased the duration of action potentials compared with those of control and heat-shock neurons (Fig. 5C; two-way ANOVA with Tukey multiple pairwise comparisons, $F=5.99$, d.f.=2, $P<0.05$). To ensure that differences in duration did not result from differences in amplitude, action potentials were matched for amplitude and duration was compared; differences in duration were

independent of amplitude (not shown). Heat shock and anoxia both decreased the amplitude of action potentials (Fig. 5D; two-way ANOVA with Tukey multiple pairwise comparisons, $F=9.15$, d.f.=2, $P<0.05$) with no difference between the two stresses. Action potential amplitude in heat-shock and anoxic neurons was also less sensitive to increases in temperature. A statistically significant interaction between treatment (i.e. control, heat shock, anoxia) and temperature was present for action potential amplitude indicating that the effect of the different treatments depends on the temperature (two-way ANOVA with Tukey multiple pairwise comparisons, $F=2.34$, d.f.=6, $P<0.05$), with the greatest differences observed at lower temperatures.

Tolerance to anoxic stress

To determine whether a prior heat stress protects locusts from a subsequent anoxic stress, control and heat-shock locusts were exposed to anoxic conditions for 1–6 h, and time to recover was recorded (Fig. 6). Time to recover after anoxia was significantly dependent upon exposure time in both control and heat-shock locusts (two-way ANOVA, $F=64.42$, d.f.=5, $P<0.001$). Heat shock significantly affected locust sensitivity to anoxia (two-way ANOVA, $F=12.82$, d.f.=1, $P<0.001$). There was also a significant interaction between treatment (i.e. control or heat shock) and exposure to anoxia

Fig. 5. The effects of heat shock and anoxia on the thermosensitivity of action potentials recorded from locust forewing motoneurons. (A) Thermosensitivity of action potential latency in control (filled circles) (N=6), heat-shock (stippled circles) (N=9) and anoxia (open circles) (N=7) neurons. Latency in heat-shock neurons is significantly shorter than latency in control and anoxic neurons (two-way ANOVA with Tukey multiple pairwise comparisons, F=6.74, d.f.=2, P<0.05). No value for anoxia neurons at 45 °C is shown because action potentials remaining at this temperature occurred spontaneously. (B) Thermosensitivity of action potential time to peak in control (filled circles) (N=6), heat-shock (stippled circles) (N=9) and anoxia (open circles) neurons (N=8). Time to peak in heat-shock neurons is significantly different from that of both control and anoxia neurons (two-way ANOVA with Tukey multiple pairwise comparisons, F=7.02, d.f.=2, P<0.05). (C) Thermosensitivity of action potential duration in control (filled circles) (N=6), heat-shock (stippled circles) (N=9) and anoxia (open circles) (N=9) neurons. Action

potentials in anoxia neurons have significantly longer durations than those of both control and heat-shock neurons (two-way ANOVA with Tukey multiple pairwise comparisons, F=5.99, d.f.=2, P<0.05). (D) Thermosensitivity of action potential amplitude in control (filled circles) (N=6), heat-shock (stippled circles) (N=9) and anoxia (open circles) (N=6) neurons. Amplitude in heat-shock and anoxia neurons is significantly smaller than that in control neurons (two-way ANOVA with Tukey multiple pairwise comparisons, F=9.15, d.f.=2, P<0.05). Statistical tests include data only from temperatures between 22 and 35 °C. At higher temperatures, the majority of control action potentials failed and, consequently, there were not enough data points for statistical comparisons at temperatures above 35 °C. Values are means ± S.E.M.
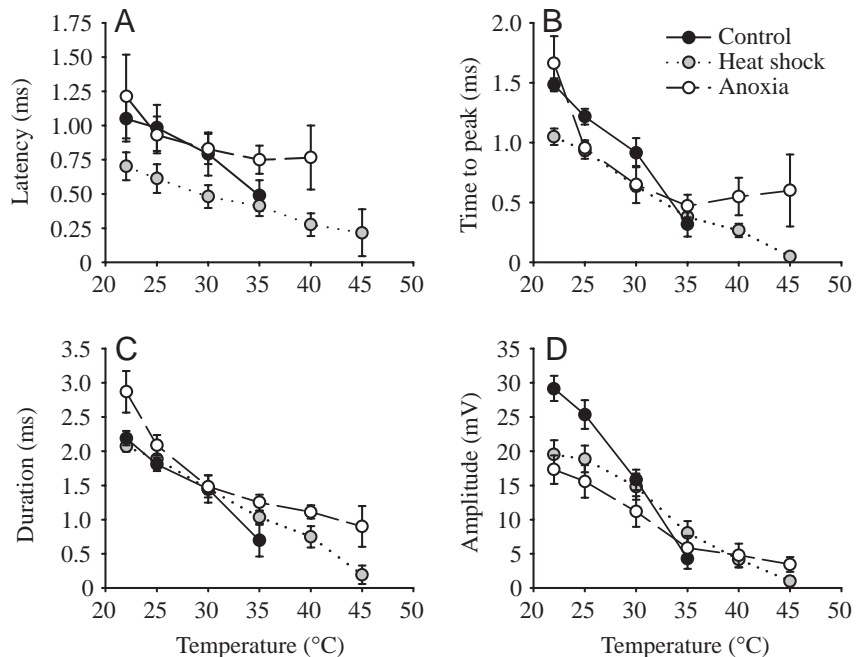
(two-way ANOVA, F=4.58, d.f.=5, P=0.001). There were no differences in the time to recover after 1, 2 and 3 h of exposure to anoxic conditions (Fig. 6). However, after 4 and 5 h of anoxia, locusts previously exposed to heat shock took a significantly longer time to recover (Fig. 6) (two-way ANOVA with Tukey multiple pairwise comparisons, F=4.58, d.f.=5, P=0.001). There was no significant difference in time to recover after 6 h under anoxia. However, two of the seven heat-shock animals died after this treatment, whereas no control animals died. This finding suggests that the cross-tolerance between different stressors observed previously (Fig. 5) (i.e. prior anoxia confers thermoprotection) occurs only when the first stress is anoxic stress and the second stress is high temperature.

Fig. 6. The effect of prior heat shock on time to recover (i.e. time to self-right, see text for details) of whole locusts after exposure to anoxia. Heat shock significantly altered locust sensitivity to anoxia (two-way ANOVA, F=12.82, d.f.=1, P<0.001). Values are means + S.E.M. Exposure time of locusts to anoxia had a significant effect on time to recover (two-way ANOVA, F=64.42, d.f.=5, P<0.001). There was also a significant interaction between experimental treatment (i.e. control or heat shock) and time under anoxic conditions (two-way ANOVA, F=4.58, d.f.=5, P=0.001). Compared with controls, heat-shocked locusts took longer to recover after 4 h and 5 h under anoxic conditions (two-way ANOVA with Tukey pairwise multiple-comparison, F=4.58, d.f.=5, P=0.001). All columns represent data from seven animals with the exception of heat-shock animals at 6 h, where N=5 because two animals died.

Bioenergetic status: mitochondrial respiration and metabolite analysis

Heat shock had no effect on the properties of isolated mitochondrial respiration (Table 1). Flight muscle contributed three times more mass than fat body and possessed twice the [Arg]+[ArgP] pool. The combined effects of pool size and relative mass imply that 80–90 % of the metabolites measured in whole thoraces are derived from thoracic muscle. Heat shock also did not alter the bioenergetic ratio [ArgP]/([Arg]+[ArgP]) assayed before anoxia (Fig. 7A) or before exercise (Fig. 7B,I).
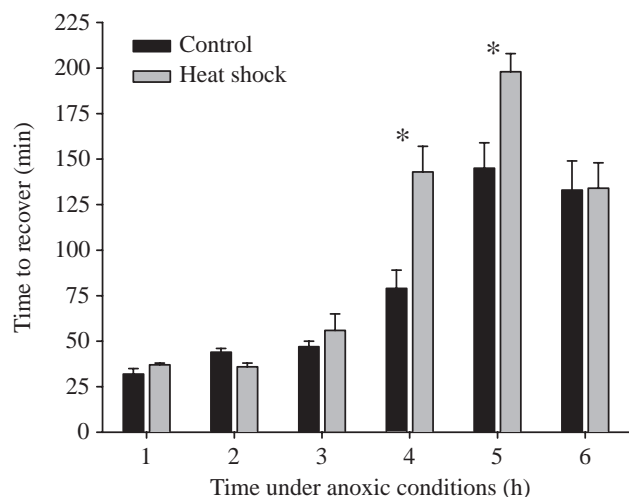
Table 1. *The effect of* in vivo *heat-shock treatment on the respiratory efficiency of mitochondria isolated from flight muscle of the migratory locust* Locusta migratoria

Treatment	Respiratory rate (nmol O_2 min^{-1} unit^{-1} CS activity)			RCI	N
	State IV	State III	State IV+		
Control	4.5±0.9	81.6±4.3	5.3±1.3	18.1	7
Heat shock	3.5±0.7	88.3±8.3	4.5±0.2	25.3	7

Measurements were conducted at 25 °C.

Values are means ± S.E.M.

All rates were obtained with 2.0 µmol pyruvate and 0.5 µmol malate as the substrates. State IV respiration is the respiration rate prior to the addition of ADP. State III respiration rate is that measured after the addition of 0.5 µmol of ADP. State IV+ is the respiration rate after the depletion of ADP.

RCI, respiratory control index; CS, citrate synthase.

After 1 h under anoxic conditions, control animals had a lower arginine charge, indicating that anoxia alone altered the bioenergetic state (Fig. 7A) (*t*-test, $t=2.79$, $P=0.03$, d.f.=6). Similarly, control + recovery and heat-shock animals had lower arginine charges (Fig. 7A) (three-way ANOVA with Tukey pairwise multiple-comparison, $F=17.22$, $P<0.001$, d.f.=4). In contrast, heat-shock + recovery animals had ratios similar to basal levels in control locusts that had not experienced any anoxic stress (Fig. 7A). After 2 h under anoxia, all groups had [ArgP]/([Arg]+[ArgP]) ratios that were almost or had been restored to pre-stress levels (i.e. similar to control values at 0 h under anoxia). Heat-shock + recovery animals after 2 h under anoxia had the highest levels of [ArgP]/([Arg]+[ArgP]). These results suggest that differences in whole-animal recovery time after anoxic stress (Fig. 6) (4 h

Fig. 7. The effects of prior heat shock, anoxia and exercise on the bioenergetic status of locust flight muscle as measured by the ratio [ArgP]/([ArgP]+[Arg]). (A) Heat shock alone did not disrupt [ArgP]/([ArgP]+[Arg]) (*t*-test, $t=-2.05$, $P=0.09$, d.f.=6). [ArgP]/([ArgP]+[Arg]) ratios were perturbed after 1 h under anoxic conditions in control (*t*-test, $t=2.79$, $P=0.03$, d.f.=6), control + recovery and heat-shock animals (three-way ANOVA with Tukey multiple pairwise comparison, $F=17.22$, $P<0.001$, d.f.=4). [ArgP]/([ArgP]+[Arg]) ratios indicated that energy reserves in all groups were restored after 2 h under anoxia. [ArgP]/([ArgP]+[Arg]) ratios increased after 1 h under anoxia despite animals being under constant anoxic conditions. The line through the control value at 0 h indicates the basal level of [ArgP]/([ArgP]+[Arg]). (B) I: heat shock alone did not significantly affect [ArgP]/([ArgP]+[Arg]). II: there was no significant difference between control animals kept at room temperature for 4 h and heat-shocked animals that had a recovery period of 1 h at room temperature. III: exercise resulted in a significant reduction in the [ArgP]/([ArgP]+[Arg]) ratio in the flight muscle of animals that had been previously subjected to heat shock. Values are means + S.E.M. ($N=7-10$). Asterisks indicate a significant difference between control and heat-shocked animals ($P<0.05$). A horizontal bar indicates no significant difference.

and 5 h under anoxic conditions) do not result from disturbances in energy reserves, which are restored to normal levels after only 2 h of stress (Fig. 7A).

Measurement of metabolites from heat-shock animals following a 1 h recovery period revealed no significant differences between heat-shock and control animals that had been kept at room temperature for 4 h (Fig. 7B, II). Flight led to a 45 % reduction in [ArgP]/([Arg]+[ArgP]) ratios in animals that had been previously heat-shocked. Exercise alone had no significant effect on [ArgP]/([Arg]+[ArgP]) (Fig. 7B, III).

Discussion

Ion channel modulation has been observed in the response to anoxia in a variety of different animals. In mammals, acute anoxia results in an increase in outward K^+ conductance (Fujiwara et al., 1987) and a decrease in Na^+ current (O'Reilly et al., 1997). In contrast, anoxia-tolerant turtles respond to acute anoxia with a decrease in K^+ current (Chih et al., 1989) and decrease in Na^+ channel density (Perez-Pinzon et al., 1992). During anoxia, the membrane potential changes observed in insect neurons are probably the result of a complex cascade of events, ultimately leading to changes in K^+ currents.
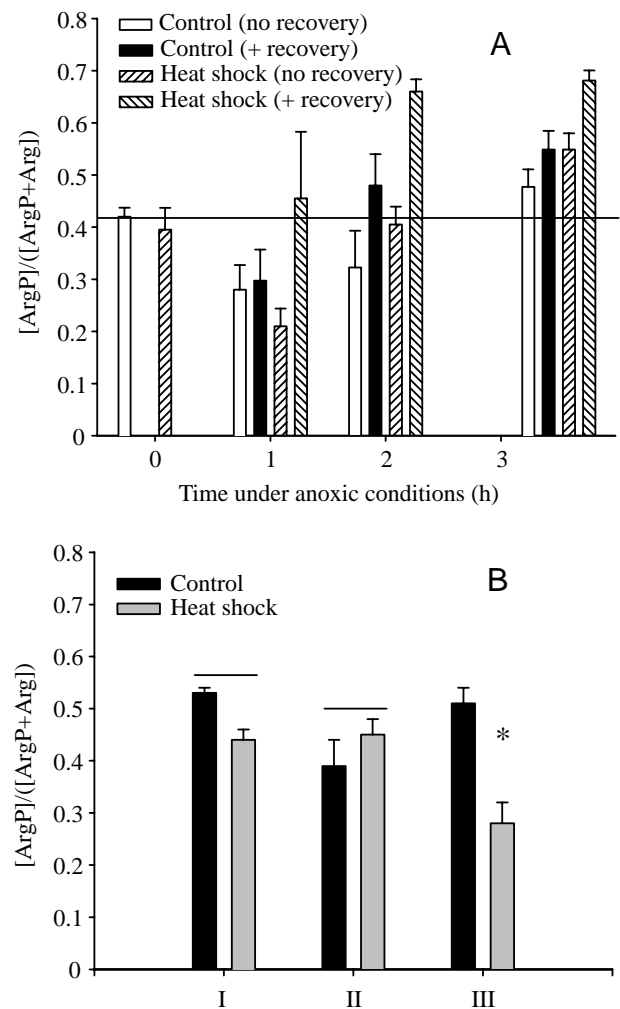
In our experiments, outward whole-cell K$^+$ currents in neuronal somata were significantly reduced by prior exposure to anoxia. There was a decrease in current amplitude resulting from a 25 % decrease in maximal whole-cell conductance following anoxia. There were, however, no changes in current activation potentials; they remained between –40 and –30 mV, and the activation curves of anoxia-treated animals did not differ from those of control animals. Furthermore, the proportion of current that was inactivated by the end of the current step did not differ for control and anoxia-pre-treated animals (10 and 12 %). This suggests that, for both control and anoxia-treated locusts, fast-inactivating currents were not a major component of the total whole-cell outward current. Taken together, the results of this study suggest that there were no changes in the types of channel involved in the whole-cell outward K$^+$ current. Instead, the amount of current being conducted through these channels changed as a consequence of prior exposure to anoxia.

One possible explanation for this result is that K$^+$ channel modulations, perhaps due to conformational changes mediated by phosphorylation status (e.g. Fadool and Levitan, 1998) in the channel proteins, resulted in a decrease in single-channel conductance. This would result in an overall reduction in the outward K$^+$ current of the cell. Alternatively, the number of active K$^+$ channels that contribute to whole-cell outward current may decrease. However, the modulation of K$^+$ channels by phosphorylation is a highly conserved phenomenon (Hille, 1992), and levels of protein tyrosine kinase are upregulated by oxygen/glucose deprivation in rats (Cayabyab et al., 2000). In addition, preliminary results in the locust suggest that increases in haemolymph serotonin levels in response to stress can decrease K$^+$ currents (J. K. Lee and R. M. Robertson, unpublished observations), suggesting that the observed changes in current are mediated by the effects of serotonin on single-channel activity.

The reduction in steady-state current as a result of anoxia is similar to that observed after heat shock (Ramirez et al., 1999), consistent with the thesis that there is a general electrophysiological response to cellular stress. However, the differences observed in the inactivation characteristics of K$^+$ currents following heat shock in locust neurons (Ramirez et al., 1999) were not seen following anoxia. In hypoxia-tolerant turtle neurons, efflux of K$^+$ is depressed by adenosine modulation during anoxia (Pek and Lutz, 1997), and in heat-shocked locusts, K$^+$ currents recorded from the soma of neurons are reduced and inactivate rapidly (Ramirez et al., 1999). It has been suggested that depressed K$^+$ currents may be a strategy for energy conservation during stress (Weckström and Laughlin, 1995) since less ATP would be required to maintain appropriate transmembrane ionic concentrations. Thus, our data most strongly support a protective strategy whereby energy is conserved by restricting ion flow and reducing the energetic demand required to maintain ion gradients. Further discussion of the protective roles played by K$^+$ current reduction can be found in Wu et al. (2001).

We measured action potentials from motoneurons during a subsequent heat stress in isolated thoracic ganglia preparations. Prior heat shock resulted in electrophysiological thermotolerance as demonstrated by the increased heat-induced failure temperature of action potential generation in motoneurons. Such thermotolerance is consistent with previous studies demonstrating that heat shock can induce thermotolerance of synaptic transmission (Dawson-Scully and Robertson, 1998) and neuromuscular transmission (Barclay and Robertson, 2000; Karunanithi et al., 1999). Action potential signalling is also protected by prior heat shock in a semi-intact locust model (Wu et al., 2001).

Anoxia treatment was found to have long-term neuroprotective effects similar to heat shock. Prior anoxia increased the upper temperature limit for generation of an action potential by more than 4.5 °C. The observed increases in the upper temperature limit for generation of an action potential would have significant consequences for preserving behaviour at high temperatures. This is particularly true for the locust, given that insects can experience hypoxia when they close their spiracles to prevent water loss (Rourke, 2000; Lighton, 1991; Lighton and Garrigan, 1995) and that *L. migratoria* are native to the hot, semi-arid regions of Africa (Chapman, 1976; Uvarov, 1966).

In addition to increasing the upper temperature limit for action potential generation, both heat-shock and anoxia treatment altered action potential characteristics. After heat shock, action potentials had smaller amplitudes but there was no difference in action potential duration. A previous study using a semi-intact locust preparation found that heat shock induced an increase in action potential duration (Wu et al., 2001). One possibility for this discrepancy in action potential duration after heat shock between previous results using the semi-intact preparation and these results using an isolated ganglia preparation is that the prolonged dissection and subsequent isolation may cause a stress. Such a stress may activate cellular changes similar to those observed after heat shock, including the reduction of outward K$^+$ currents that may account for the differences in action potential duration (Ramirez et al., 1999). Consistent with this idea is the increase in the absolute value of duration in control cells after isolation. In semi-intact preparations, duration at half-amplitude measured at 25 °C was 1.43±0.08 ms (mean ± S.E.M., N=8) (Wu et al., 2001) compared with 1.81±0.09 ms (mean ± S.E.M., N=6) in the isolated preparation. A variety of stressors cause modulation of K$^+$ currents, and a reduction in outward K$^+$ currents may be a conserved response to cellular stress. Thus, if heat shock and isolation of the ganglia trigger similar cellular responses, some changes induced by heat shock may be masked in the isolated preparation.

In cells treated with anoxia, action potential amplitude and duration were both altered. Anoxia decreased action potential amplitude and increased action potential duration. Similar changes in action potential amplitude and duration have been observed previously in response to acute hypoxic stress, and it has been suggested that stress-induced changes in action potential shape result from modulation of Na$^+$ and K$^+$ currents

in *Drosophila melanogaster* neurons (Gu and Haddad, 1999). The decrease observed in action potential amplitude after both heat shock and anoxia in our experiments could be attributed to an inhibition of Na^+ currents (Gu and Haddad, 1999; Cummins et al., 1994). The increase in action potential duration after anoxia, in contrast, is most probably due to a decrease in Ca^{2+}-activated K^+ currents, which are responsible for restoring the membrane potential and action potential after-hyperpolarization (Mills and Pitman, 1999; Gu and Haddad, 1999; Hille, 1992).

Proper interpretation of the observed reduction in outward K^+ currents and how it might relate to increases in action potential duration must consider that the variables were recorded from different cells and from different regions of the neuron. Nonetheless, we suggest that these electrophysiological changes contribute to the ability of anoxia-preconditioned animals to maintain nervous system activity during a subsequent stress. We conclude that decreases in K^+ conductance may represent a major target of modulation during environmental stress. The cross-tolerance between anoxia and a subsequent heat stress suggests that heat shock and anoxia activate similar cellular pathways and that these pathways represent general mechanisms for adaptation to a diverse variety of stressors.

Heat shock has been shown to induce cross-tolerance and thermotolerance in a number of organisms, and there is ample evidence that heat shock has protective effects on neuronal tissues subsequently exposed to anoxia (Marcuccilli and Miller, 1994; Sharp et al., 1999; Ikeda et al., 1999; Wada et al., 1999; Feder and Hofmann, 1999). To determine whether protection of electrophysiological events would be extended to whole-animal survival, the effect of a prior heat shock and anoxia on survival rates during a subsequent heat stress were studied. Preconditioning locusts with heat-shock or anoxia treatment induces protective effects on the ability of locusts to survive a subsequent exposure to normally lethal temperatures. Curiously, cross-tolerance occurs in one direction only in that a prior heat shock appears to make the locusts more vulnerable to a subsequent anoxic stress. In mammals, heat shock has been found to protect against neuronal hypoxic ischaemic stress, but in these studies protection was at the cellular (as opposed to organismal) level and there was a longer recovery period following the initial stress (Wada et al., 1999; Ikeda et al., 1999; Ota et al., 2000). Although both these stresses cause similar changes at the level of neural circuitry, the general responses to heat shock and anoxia in locusts are quite different. Insects respond to anoxia with a loss of function of all organs and suspension of nervous system activity (Weyel and Wegener, 1996; Wegener, 1993), resulting in complete rigid paralysis. In contrast, recordings of nervous system activity during high-temperature stress indicate that signalling continues up to near-lethal temperatures (Barclay and Robertson, 2000, 2001; Gray and Robertson, 1998; Robertson et al., 1996), that insect ventilation movements persist and that the animals remain active even if the stress persists for several hours, although at a reduced level (B. S. Wu and R. M.

Robertson, unpublished observations). These differences and the complex nature by which stress-activated pathways are integrated within the whole animal probably account for the differences in cross-tolerance reported here.

Although a general consequence of cellular stress is the disruption of bioenergetic homeostasis, bioenergetic indicators showed that heat shock alone did not cause a perturbation in energy metabolism. In contrast, the bioenergetic state of animals that had been exposed to heat shock after 1 h under anoxia was significantly affected. Results from heat-shock animals that had performed a subsequent flying exercise confirmed that heat shock renders locusts energetically vulnerable and that this is revealed only after a subsequent exposure to stress. Neither exercise alone nor heat shock alone had dramatic effects on metabolite profiles, but when heat-shocked locusts were exercised, significant effects on [ArgP]/([ArgP]+[Arg]) ratios were observed. This metabolic vulnerability following heat shock is in marked contrast to what has been termed 'induced stress tolerance', in which heat-shocked organisms are typically less sensitive to a subsequent stress. While this heat-shock treatment induces thermotolerance in neural circuitry (Dawson-Scully and Robertson, 1998; Barclay and Robertson, 2000), metabolism in muscle is rendered vulnerable. The basis for this vulnerability is not known, and we could find no indication from analysis of arginine charge or isolated mitochondria.

Anoxia caused an immediate decrease in arginine charge in control and heat-shock locusts. In both groups, arginine charge recovered after 2 h despite continued anoxic exposure. The increases in [ArgP]/([ArgP]+[Arg]) ratios that occur despite animals remaining under constant anoxic conditions suggests that, although anaerobic metabolism is minimal in insects (Wegener, 1993), it has a physiologically relevant effect when animals are exposed to anoxic conditions and significantly decrease demands for ATP. The restoration of arginine charge in control and heat-shock animals after 2 h also suggests that energy metabolism does not appear to be responsible for the observed increase in time to recover after anoxic stress observed in whole animals since bioenergetic status was restored rapidly after the initial perturbation.

Although heat-shocked locusts were not protected against anoxic stress, heat-shocked locusts that were permitted to recover after the anoxic stress had [ArgP]/([ArgP]+[Arg]) ratios that were restored to a level similar to control levels. This is perhaps a reflection of pH rather than energy metabolism. Increases in pH, possibly arising from hyperventilation during the recovery period under normoxic conditions, could drive the ArgK equilibrium towards ArgP production. However, control locusts that have recovered from anoxia after 1 h do not have an increased arginine charge. Thus, hyperventilation may affect heat-shock animals differently or it may not be causing increases in [ArgP]/([ArgP]+[Arg]). It is also possible that heat-shock animals respond differently during post-anoxia recovery as oxidative phosphorylation resumes and a positive energy balance is gradually restored. In this scenario, heat-shocked locusts, unlike control animals after anoxia, do not

assume a normal posture until bioenergetic status represented by [ArgP]/([ArgP]+[Arg]) is completely restored. Perhaps heat shock preconditions locusts to respond to an anoxic stress by altering their metabolic rate. Such a change could be induced by a transition into an energy-conserving state.

Control locusts, like other insects, initially increase production of ATP in response to anoxia in an attempt to keep up with increasing demand. Heat-shock animals will also transiently fill increasing demand (for heat-shock protein transcription, translation and ATPase activity) but, since they are already conserving energy, they will change survival strategies more readily. Unlike anoxia-tolerant vertebrates, insects have very minimal anaerobic metabolism abilities (Wegener, 1993). However, they are able to survive anoxia by utilizing a strategy that minimizes energy consumption. Thus, it is possible that heat-shock locusts are closer to a threshold of anoxic stress that will trigger the cessation of all unnecessary energy consumption. Consequently, when oxygen becomes available again, they are able to restore their pool of available energy rapidly since demand has already been minimized. Control animals have not yet activated the cellular signals that cause changes from an increase in ATP production to a conservation of energy strategy and, therefore, have a delayed response in restoring their levels of ArgP.

Although temperature- and water-dependent physiological challenges exist for insects that live in hot arid environments, they nonetheless thrive under such conditions. A strategy that insects use to minimize water loss is behavioural modification of spiracular ventilation (Rourke, 2000; Lighton and Garrigan, 1995; Lighton et al., 1993). Closing the spiracles minimizes water loss but can cause short-term hypoxia and hypercapnia (Rourke, 2000). Such behavioural modifications that induce hypoxia can be exacerbated by high environmental temperatures (Rourke, 2000; Lighton and Garrigan, 1995; Lighton et al., 1993; Schmidt-Nielsen, 1990; Mellanby, 1934). *L. migratoria* are native to the hot semi-arid regions of Africa where temperatures often exceed 40 °C (Chapman, 1976; Uvarov, 1966). Thus, it is likely that locusts will encounter both high temperature and hypoxic stress and, with respect to their flight circuitry, modulation of electrophysiological events that permit an increase in the upper range of operant temperatures would be advantageous. Our results suggest that heat shock and anoxic stress activate similar cellular pathways that result in thermotolerance of action potential signalling and whole-animal survival. However, the induced cross-tolerance we observed was unidirectional because heat shock did not protect against a subsequent anoxic stress. This finding is important, but at present it could be attributed either to activation of different pathways for protection or to differential activation of identical pathways. Distinguishing these possibilities must wait until we have better ways of equating the stress treatments that the organism experiences. Nevertheless, the differences in levels of cross-tolerance may represent the locusts' response to relative risk associated with heat and anoxic stress in their natural habitat. Given the locust's ability to decrease energy consumption substantially during anoxia, it is likely that desiccation at high temperatures poses a larger threat to its survival; consequently, the locust would derive a greater benefit from induced thermotolerance than from induced tolerance to anoxia.

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