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Controlling anoxic tolerance in adult *Drosophila via* the cGMP–PKG pathway

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SUMMARY

In this study we identify a cGMP-dependent protein kinase (PKG) cascade as a biochemical pathway critical for controlling low-oxygen tolerance in the adult fruit fly, *Drosophila melanogaster*. Even though adult *Drosophila* can survive in 0% oxygen (anoxia) environments for hours, air with less than 2% oxygen rapidly induces locomotory failure resulting in an anoxic coma. We use natural genetic variation and an induced mutation in the *foraging* (*for*) gene, which encodes a *Drosophila* PKG, to demonstrate that the onset of anoxic coma is correlated with PKG activity. Flies that have lower PKG activity demonstrate a significant increase in time to the onset of anoxic coma. Further, *in vivo* pharmacological manipulations reveal that reducing either PKG or protein phosphatase 2A (PP2A) activity increases tolerance of behavior to acute hypoxic conditions. Alternatively, PKG activation and phosphodiesterase (PDE5/6) inhibition significantly reduce the time to the onset of anoxic coma. By manipulating these targets in paired combinations, we characterized a specific PKG cascade, with upstream and downstream components. Further, using genetic variants of PKG expression/activity subjected to chronic anoxia over 6 h, ~50% of animals with higher PKG activity survive, while only ~25% of those with lower PKG activity survive after a 24 h recovery. Therefore, in this report we describe the PKG pathway and the differential protection of function *vs* survival in a critically low oxygen environment.

Key words: *Drosophila*, PKG, anoxia, cGMP, hypoxia, locomotion.

INTRODUCTION

The fruit fly *Drosophila melanogaster* is an anoxia-tolerant animal, where it can survive in low levels of oxygen for hours without pathology (Wingrove and O'Farrell, 1999; Haddad, 2006). One of the possible methods by which the fruit fly deals with such harsh environmental conditions over this period of hours seems to be dependent on the sudden onset of an anoxic coma when oxygen levels drop below 2% (Haddad, 2006). Therefore, unlike mammals that exhibit cell death within minutes of critically low oxygen, fruit flies are an ideal model system for examining genes and molecular pathways responsible for the tolerance of anoxic stress without the complication of pathological incident during investigations of acute oxygen deprivation.

The ability to control an animal's tolerance to low oxygen could mitigate pathologies such as stroke and neurodegenerative disease (Ogawa et al., 2007). In the past, *foraging* (*for*) alleles have been linked to natural variation in numerous phenotypes including food-searching behaviors (Osborne et al., 1997; Ben-Shahar et al., 2002; Raizen et al., 2006), learning and memory (Scheiner et al., 2004; Kaun et al., 2007a; Mery et al., 2007), and most recently, the thermotolerance of neural function (Dawson-Scully et al., 2007). In a natural population, ~70% of flies demonstrate higher cGMP-dependent protein kinase (PKG) activity and express the rover allele of the *for* gene, where 30% have lower PKG activity and express the sitter allele (Osborne et al., 1997). Under prolonged hypoxia, rover embryos and larvae survive significantly longer than sitters (Wingrove and O'Farrell, 1999). Therefore, based on the roles of PKG in modulating responses to other stresses such as food deprivation or thermal stress, we hypothesized that natural variation in *for* would also modulate acute low-oxygen tolerance during locomotory behavior and animal survival in adult *D. melanogaster*.

In the present study we demonstrate for the first time that natural variation in tolerance of behavior to acute hypoxia between the rover and sitter alleles of the *foraging* gene, involves components of the PKG pathway in *D. melanogaster*; and is inversely related to the tolerance of survival during prolonged anoxia.

MATERIALS AND METHODS

Using both genetics and pharmacology, we examined the roles and order of function of additional cGMP-dependent signaling molecules in modulating acute low-oxygen sensitivity of locomotory failure. To do this, we imposed acute hypoxia (<0.2% O_2 within 5 min) by displacing environmental oxygen with the inert gas argon and then measured time to failure of locomotion (anoxic coma), which can be observed in less than 2% atmospheric oxygen (Haddad, 2000). We also used a novel but reliable method of pharmacological volatilization as a means to manipulate, *in vivo*, the activity of target enzymes and molecules such as PKG. Flies were frozen immediately after each trial, and heads were dissected and examined for PKG activity, as an indicator for activity levels in the brain (Belay et al., 2007; Kaun et al., 2007b). Lastly, using adult flies we tested what has been previously shown in embryos and larvae (Wingrove and O'Farrell, 1999), i.e. the effect of prolonged anoxia (6 h) on animal survival, after a 24 h recovery.

Fly stocks

Two naturally occurring strains of adult *Drosophila melanogaster* Meigen were used in this study: a rover strain homozygous for the for^R allele (high PKG activity), and a sitter strain homozygous for for^s (low PKG activity). These strains are isogenized natural polymorphisms of the *foraging* gene, located on the second chromosome (Sokolowski, 1980; Fitzpatrick et al., 2007). Additionally, the sitter mutant strain for^{s2}, which was previously

generated in the laboratory, was also utilized. This strain has a rover genetic background with a mutation at *for* leading to PKG activity/transcript levels lower than that observed in sitters (Pereira and Sokolowski, 1993). All flies were reared in the same fashion; 12 h:12 h light:dark cycle with lights on at 08:00 h, equal density (approximately 100 flies in a 170 ml plastic culture bottle containing 40 ml of a standard yeast–sucrose–agar medium), and same age (5–9 days old adults at testing time) in an incubator at 25°C. Flies that were used in this study were not exposed to anesthesia for at least 24 h before trials.

Locomotory tolerance assay during acute hypoxia

Ten flies (separated into males or females) from each of the three strains [rover (for^R), sitter (for^s) and for^{s2}] were selected and placed in three separate vials with food at least 24 h before each experiment. At the time of the experiment, flies were emptied into 10 ml empty test vials covered with permeable sponge caps of equivalent thickness, allowing for consistent gas exchange. These test vials were then placed into a 600 ml beaker, which was covered with parafilm, creating a closed chamber with an escape hole in the parafilm (1 cm diameter). The purpose of this hole is to allow the lighter normal air (compared with argon which is heavier) to escape from the top of the beaker. The parafilm top was punctured with a needle connected to a gas tank (by a plastic hose), through which the inert gas argon was expelled. Preliminary work was done with pure nitrogen, where no significant differences between the uses of the two gases were observed (data not shown). Argon gas was chosen to displace normoxic air over nitrogen in these studies since previous work demonstrated that nitrogen-treated *Drosophila* had an unusually prolonged recovery time from the onset of chill coma (Nilson et al., 2006).

The needle tip was fastened to the bottom of the beaker and inserted into a larger sponge of similar texture to the vial sponge caps to ensure that gas expelled from the needle was dispersed equally throughout the beaker. Also, to ensure that all test vial positions in the beaker received equal gas flow, test vial positions were alternated every trial to reduce variability.

The experiment required exposing the test vials with 10 flies in a vial within the container to pure argon gas expelled from the tank at a flow rate of 600 cc min^{-1} ±5%. Time of behavioral failure was recorded by observing the fly undergo a seizure, which was quickly followed by an anoxic coma. A novel computer program entitled Multi-Arena-Multi-Event-Recorder (MAMER, freely available at http://cfly.utm.utoronto.ca/MAMER), developed by Dr Craig A. L. Riedl, was used to record neural failure time for each individual fly. This was accomplished by starting the timer on the program at the initiation of gas flow. The observer would then type ‘1’ if a fly failed in vial 1 (where 10 flies reside), ‘2’ for fly failure in vial 2, and ‘3’ for observed failure in vial 3. The program recorded these times of failure for each individual fly within the vials and then computed a mean failure time for each vial (each vial has 10 flies but gives a mean value for N=1). The averaged failure times represent the N data in the figures, and successfully distinguished failure times among the different strains. Upon failure of all flies, the vials were placed in a freezer at –20°C to preserve PKG levels at time of behavioral failure. These flies were later subjected to PKG enzyme activity assays to measure PKG enzyme levels. Males and females were tested separately, and no significant differences were found between any of the genotypes or treatments and therefore the data was pooled (P>0.05).

Survival assay during prolonged anoxia

Ten flies (separated into males or females) from each of the three strains [rover (for^R), sitter (for^s) and for^{s2}] were selected and placed in three separate vials with food at least 24 h before each experiment. These test vials were then placed into a 1000 ml beaker, which was covered with parafilm, creating a closed chamber with an escape hole (1 cm diameter). The parafilm top was punctured with a needle connected to a gas tank (by a plastic hose), through which the inert gas argon was expelled.

The needle tip was fastened to the bottom of the beaker and inserted into a larger sponge of similar texture to the vial sponge caps to ensure that gas expelled from the needle was dispersed equally throughout the beaker. The gas was turned on at a flow of 600 cc min^{-1} and left for 6 h. Vials were removed after the experiment and placed in to a 25°C 12 h:12 h light:dark cycle for 24 h, and animals alive were counted.

Pharmacological manipulation

The same behavioral assay described above was employed; however, flies were pre-treated with chemical agents to observe their effects on tolerance to anoxic stress. The pharmacological behavioral assays were conducted on the sitter strain because of its intermediate tolerance when compared with rovers and the sitter mutant (Fig. 1). Adult sitters were exposed to various drugs predicted to have an effect on targets involved in the PKG pathway. These drugs included (from Sigma Aldrich, St Louis, MO, USA):

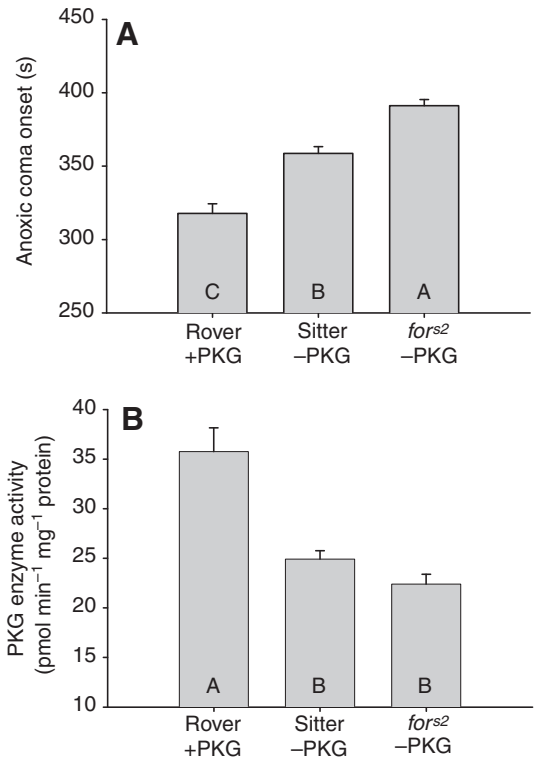

Fig. 1. Anoxic coma onset is modulated by cGMP-dependent protein kinase (PKG) activity through natural variation of the *foraging* gene. (A) Time to anoxic coma onset (time to failure) in adult *Drosophila melanogaster* during acute hypoxia by air displacement using pure argon gas. (B) PKG activity was assayed from the heads of animals in A. All vertical bar charts are shown as means ± s.e.m. Significant differences were established with P<0.05, where letters that differ on the graphs signify statistical groupings.

10 mmol l^{-1} T0156 (a cGMP-specific phosphodiesterase-5 inhibitor), 10 mmol l^{-1} 8-bromo-cGMP (a PKG activator), 1 mmol l^{-1} KT5823 (a PKG inhibitor), 1 mmol l^{-1} cantharidin (a PP2A inhibitor) and 200 mmol l^{-1} DCA (a K^+ channel activator). All drugs were solubilized in dimethyl sulfoxide (DMSO), where flies treated with only DMSO were used as a sham controls. We developed a novel assay to administer these drugs to whole adults *in vivo* through volatilization, since we used concentrations of the drug at 1000-fold (μmol l^{-1} *vs* mmol l^{-1}) concentrations used *in vitro* (Dawson-Scully et al., 2007). 10 μl of drug solution was applied to a crushed Kim wipe (VWR International, West Chester, PA, USA) at the bottom of each test vial. An additional Kim wipe was crushed over the top of this to prevent direct contact of the fly on the solution. Ten flies were then placed in the vial, which was capped with a semi-permeable flug and covered with a cut-out finger of a large latex glove to prevent chemical vapours from escaping in the dark. The flies were subjected to the drug for 1 h prior to each behavioral assay.

Drug combinations

Drug combinations were employed to observe the effects of activating and/or inhibiting various participants in the PKG pathway simultaneously to determine downstream and upstream targets. Here 20 μl of DMSO was used as a sham control, and combinations of two pharmacological treatments were added as two separate 10 μl aliquots. The same drugs and protocol described above were used in this assay. The drug combinations tested were: 10 mmol l^{-1} 8-bromo-cGMP/1 mmol l^{-1} KT5823, 10 mmol l^{-1} 8-bromo-cGMP/1 mmol l^{-1} cantharidin, 10 mmol l^{-1} 8-bromo-cGMP/10 mmol l^{-1} T0156, 10 mmol l^{-1} 8-bromo-cGMP/200 mmol l^{-1} DCA, 1 mmol l^{-1} KT5823/1 mmol l^{-1} cantharidin, 1 mmol l^{-1} KT5823/10 mmol l^{-1} T0156, 1 mmol l^{-1} KT5823/200 mmol l^{-1} DCA, 1 mmol l^{-1} cantharidin/10 mmol l^{-1} T0156, 1 mmol l^{-1} cantharidin/200 mmol l^{-1} DCA and 10 mmol l^{-1} T0156/200 mmol l^{-1} DCA.

PKG enzyme activity assays

PKG enzyme activity assays were conducted according to the procedure outlined in Kaun et al. (Kaun et al., 2007a; Kaun et al., 2007b). Adult *Drosophila* were decapitated and the heads were homogenized in 25 mmol l^{-1} Tris (pH 7.4), 1 mmol l^{-1} EDTA, 2 mmol l^{-1} EGTA, 5 mmol l^{-1} β-mercaptoethanol, 0.05% Triton X-100 and protease inhibitor solution (Roche Diagnostics, Indianapolis, IN, USA). Following microcentrifugation for 5 min, the supernatant was removed and those supernatants containing equal amounts of total protein were examined for PKG enzyme activity. The reaction mixture contained the following substances: 40 mmol l^{-1} Tris-HCl (pH 7.4), 20 mmol l^{-1} magnesium acetate, 0.2 mmol l^{-1} [γ^{32}P]ATP (500–1000 c.p.m. pmol^{-1}), 113 mg ml^{-1} heptapeptide (RKRSRAE), 3 mmol l^{-1} cGMP and a highly specific inhibitor of cAMP-dependent protein kinase. The next step of the procedure involved incubating the reaction mixtures at a temperature of 30°C for 10 min, followed by ending the reaction by spotting 70 μl of the reaction mixture onto Whatman P-81 filters (VWR International). To remove any unreacted [γ^{32}P]ATP, these spots were then soaked with 75 mmol l^{-1} H_3PO_4 for 5 min and washed three times with 75 mmol l^{-1} H_3PO_4. Before quantifying enzyme activity, filters were rinsed with 100% ethanol and air dried. To calculate PKG enzyme activity, counts were taken in a Wallac 1409 Liquid Scintillation Counter (Pegasus Scientific, Rockville, MD, USA) using universal scintillation cocktail (ICN). PKG activity was presented in the figures as pmol of ^{32}P incorporated into the substrate per minute per mg of protein (pmol min^{-1} mg^{-1} protein).

Statistics

Data were analyzed using one-way and two-way analysis of variance (ANOVA) followed by a *post-hoc* Multiple Comparisons test (SNK=Student–Neuman–Keul's test). In cases where normality or equal variance failed, non-parametric tests on the ANOVA on ranks were used. Significant differences were established with $P<0.05$, where letters that differed on the graphs signified statistical groupings. In behavioral trials, $N=1$ represents a trial that consisted of one vial with 10 adult flies.

RESULTS

Function: acute hypoxia and behavior

The natural alleles

To investigate the role of PKG in regulating sensitivity to acute hypoxia leading to anoxic coma, homozygous flies with different *for* alleles, either the natural rover (higher PKG activity) or sitter (lower PKG activity) alleles, or *for*^{s2}, a hypomorphic *foraging* mutant induced on a rover genetic background, were assayed for locomotion failure (see Materials and methods). Time to anoxic coma onset (time to failure) in adult *D. melanogaster* during acute hypoxia by air displacement using pure argon gas, was significantly increased in the natural allele of the *foraging* gene, sitter ($N=18$; low PKG activity), when compared with rover ($N=18$; high PKG activity; Fig. 1A). Further, *for*^{s2} ($N=18$), a *foraging* mutant in rover (low PKG activity), was also significantly resilient to acute hypoxia when compared with the two natural alleles [one-way ANOVA, $F_{(2,51)}=50.352$, $P<0.001$; Multiple Comparisons, SNK, $P<0.05$]. PKG activity was then assayed from the heads of animals in Fig. 1A, confirming that rovers exhibited high PKG activity whereas sitters and *for*^{s2} showed significantly lower PKG activity [$N=6$ for each genotype; one-way ANOVA, $F_{(2,15)}=20.360$, $P<0.001$; SNK, $P<0.05$; Fig. 1B].

Single compound exposure

We assessed whether fly PKG levels could be manipulated *in vivo* by means of using the volatilization of pharmacological agents. Previously we have used pharmacology on tissue preparations to demonstrate that cGMP, PKG and PP2A targets regulate the tolerance of synaptic transmission during acute hyperthermia at the *Drosophila* larval neuromuscular junction (Dawson-Scully et al., 2007). In the present study, we examine the role of these targets *in vivo* using adult sitter flies by depositing pharmacological agents dissolved in DMSO on a cellulose tissue, and allowing them to volatize for a few minutes in an airtight vial at room temperature (Fig. 2). In order to examine the PKG–PP2A–K^+ channel axis, we used dichloroacetate (DCA), a compound that has been shown to augment the nitric oxide (NO)/K^+ channel axis (Michelakis et al., 2003) in a number of cell types (Michelakis et al., 2002; Bonnet et al., 2007). Interestingly, previous work has also shown that, physiologically, neuronal whole-cell K^+ currents are reduced in animals that have undergone a heat shock preconditioning (Ramirez et al., 1999). This is associated with the protection of the nervous system during hyperthermic stress (Dawson-Scully and Robertson, 1998). We therefore tested the hypothesis that DCA, which is known to increase K^+ currents, will induce sensitivity of behavior to acute anoxia (Michelakis et al., 2003; Bonnet et al., 2007). We found that each treatment had a significant effect, either increasing or decreasing anoxic coma onset sensitivity [Kruskal–Wallis, $H_{(5)}=107.454$, $P<0.001$; Multiple Comparisons, Dunn's, $P<0.05$; Fig. 2A], where 1 mmol l^{-1} KT5823/(–)PKG [$N=47$; pharmacological agent/(+ activates/– inhibits)target] and 1 mmol l^{-1} cantharidin/(–)PP2A ($N=12$) demonstrated significant resilience to

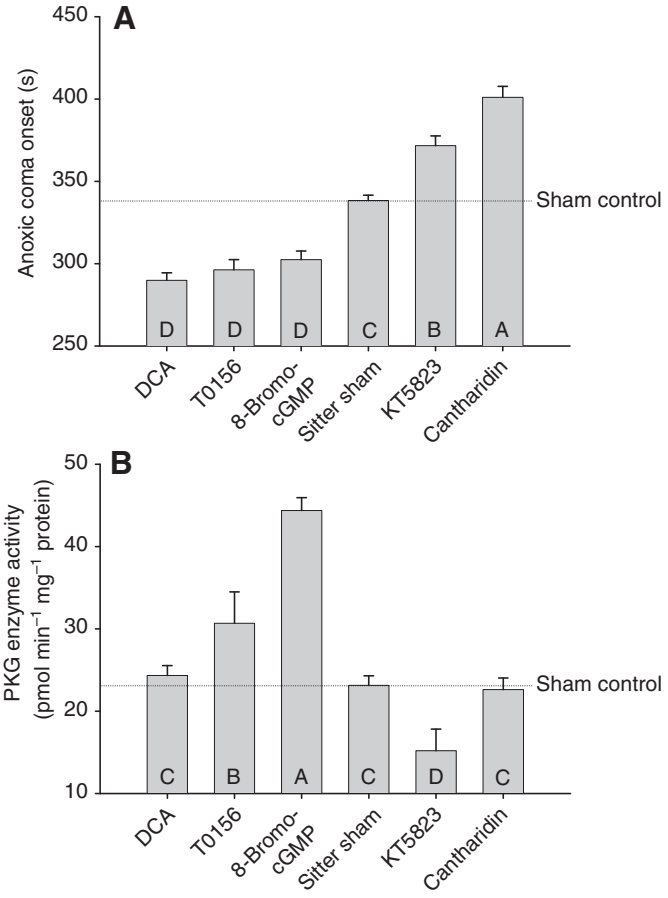

Fig. 2. *In vivo* pharmacological manipulation of various molecular targets in the cGMP-dependent protein kinase (PKG) pathway modulates time to anoxic coma onset during acute hypoxia. (A) Various volatilized pharmacological agents were used *in vivo* on the intermediate resilient allele sitter, to determine how different molecular targets modulate anoxic coma onset during acute hypoxia. (B) PKG enzyme activity assays on heads of animals derived from experiments shown in a shows that agents which could manipulate PKG either directly such as 8-bromo-cGMP/(+)PKG and KT5823/(–)PKG or indirectly such as T0156/(–)PDE5/6 (which would increase intracellular cGMP) demonstrated significant effects compared with that of the sham control [N=6 for each treatment; one-way ANOVA, $F_{(5,30)}$=20.898, P<0.001; SNK, P<0.05]. However, targets that were downstream of PKG (see Fig. 2A), such as cantharidin/(–)PP2A and DCA/(+)K^+ channels, showed no significant effects on PKG enzyme activity levels compared with sham controls (SNK, P>0.05). All vertical bar charts are shown as means ± s.e.m. Significant differences were established with P<0.05, where letters that differ on the graphs signify statistical groupings. Horizontal dotted line represents mean of sham control for ease of comparison across treatments.

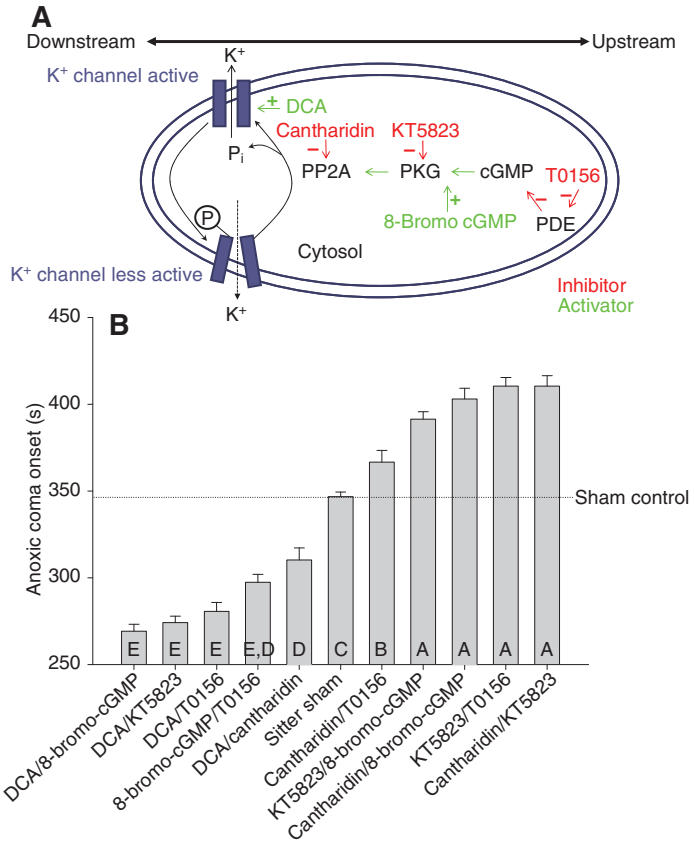

Fig. 3. Combinations of pharmacological agents that modulate time to anoxic coma onset during acute hypoxia reveal downstream and upstream molecular targets in the cGMP-dependent protein kinase (PKG) pathway. (A) Adapted from Zhou et al. (Zhou et al., 1996), this diagram and experimental design represents upstream and downstream intracellular targets for manipulating the PKG pathway partially implicated in the modulation of hyperthermic stress (Zhou et al., 1996; Dawson-Scully and Robertson, 1998). Protein phosphatase 2A (PP2A), PKG, cyclic GMP (cGMP), phosphodiesterases (PDE) and K^+ channels are shown as potential targets for pharmacological manipulation. Inhibitory compounds are shown in red with a minus (–) sign, while activators are shown in green with a plus (+) sign. The diagram shows that molecular targets and pharmacological compounds to the left are downstream of those on the right, as shown by the large double arrow at the top of the diagram. Our hypothesis states that inhibition of this pathway would result in a decrease in whole-cell K^+ channel conductance, thereby leading to increased resilience to anoxic coma onset. (B) Combinations of the pharmacological agents used, *in vivo*, shown in Fig. 2, were administered to adult *Drosophila melanogaster*, and then the animals were tested for resilience to anoxic coma onset during acute hypoxia. Vertical bar chart is shown as means ± s.e.m. Significant differences were established with P<0.05, where letters that differ on the graphs signify statistical groupings. Horizontal dotted line represents mean of sham control for ease of comparison across treatments.

anoxic coma (Dunn's, P<0.05), and 200 mmol l^{-1} DCA/(+)K^+ channels (N=15), 10 mmol l^{-1} 8-bromo-cGMP/(+)PKG (N=34) and 10 mmol l^{-1} T0156/(–)PDE5/6 (N=16) exhibited significant sensitivity to anoxic coma (Dunn's, P<0.05).

PKG enzyme activity assays on heads of animals derived from experiments shown in Fig. 2A show that agents which could manipulate PKG activity either directly such as 8-bromo-cGMP/(+)PKG and KT5823/(–)PKG or indirectly such as T0156/(–)PDE5/6 (which would increase intracellular cGMP) demonstrated significant effects compared with that of the sham control [N=6 for each treatment; one-way ANOVA, $F_{(5,30)}$=20.898, P<0.001; SNK, P<0.05; Fig. 2B]. However, targets that were downstream of PKG (see Fig. 2A), such as cantharidin/(–)PP2A and DCA/(+)K^+ channels, showed no significant effects on PKG enzyme activity levels compared with sham controls (SNK, P>0.05).

Compound combination exposure

We next used two simultaneous pharmacological treatments of the above mentioned compounds in all complementary combinations, to verify upstream and downstream components (Fig. 3A) of this proposed pathway. We investigated the hypothesis that the effects

of downstream molecular targets would override upstream targets. Similar to experiments using individual compounds (Fig. 2A), we found that each combined treatment of two agents either significantly increased or decreased anoxic coma onset sensitivity when compared with sham controls [one-way ANOVA, $F_{(10,184)}$=105.634, P<0.001; SNK, P<0.05]. As predicted, we found that pharmacological agents that inhibited or activated downstream molecular targets, as shown in Fig. 3A, directed the anoxic coma onset phenotype. For example, any time animals (Fig. 3B) were treated with a combination of drugs that included DCA, a significant decrease in resilience to anoxic coma onset was observed, when compared with sham controls (SNK, P<0.05).

Survival: prolonged anoxia and animal survival

We tested animal survival in prolonged anoxia, where we exposed adult flies with varying PKG activity (rover=high PKG, sitter and for^{s2}=low PKG) to six hours of anoxia and subsequent reoxygenation. We found that after 24 h of recovery, rovers exhibit a significant increase in the number of animals that survive compared with sitters and for^{s2} strains (Fig. 4).

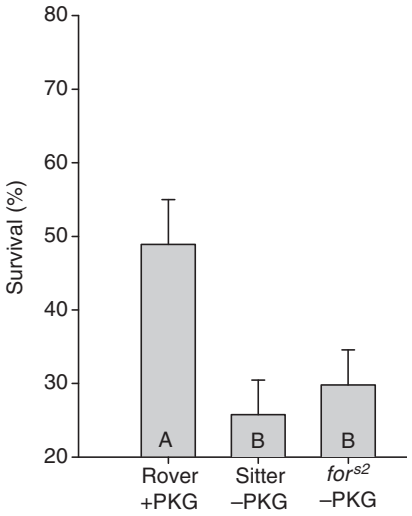

Fig. 4. Animal survival after prolonged anoxic exposure is modulated by cGMP-dependent protein kinase (PKG) activity by the *foraging* gene. Animals that genetically express increased PKG activity (+PKG) demonstrate an increase in animal survival after 6 h of anoxia. Alternatively, animals that genetically express reduced PKG activity (–PKG) demonstrate increased death. Adult 5–9 days old *Drosophila* were exposed to 6 h of anoxia, and allowed to recover for 24 h. Number of flies that survived are depicted here, where rovers (+PKG; N=29) demonstrate a significant survival rate over sitters (N=29) and for^{s2} (–PKG; N=28). Vertical bar chart is shown as means ± s.e.m. Letters A, B signify significant differences P<0.05. These were analyzed using a two-way ANOVA, $F_{(2,117)}$=12.693, P<0.001; SNK, P<0.05.

DISCUSSION

Not all animals are equally susceptible to critically low oxygen. Facultative anaerobes are evolutionarily adapted to withstand long periods without oxygen; anoxia survival tolerance of at least several hours has been established in the fruit fly *D. melanogaster* (Wingrove and O'Farrell, 1999; Haddad, 2006) while some turtles can withstand anoxia for days to months (Ultsch, 2006). These anoxia-tolerant organisms, in contrast to mammalian systems, enter a state of deep reversible hypometabolism, thereby losing neural function but maintaining a balance between energy requirements and supply by suppressing energy-demanding functions, including the release of excitatory neurotransmitters (Milton et al., 2002; Milton and Lutz, 2005) and ion flux (Sick et al., 1982; Perez-Pinzon et al., 1992; Bickler et al., 2000), which together suppress electrical activity (Fernandes et al., 1997; Gu and Haddad, 1999). Anoxia tolerance then permits survival of extended anoxia without neuronal deficit (Haddad, 2006; Kesaraju et al., 2009). In the work reported here, we demonstrate that under anoxic stress we have identified two opposing phenotypes: (1) during PKG pathway inhibition, we observe the protection of locomotion during acute hypoxia, and (2) during PKG pathway activation, we observe the protection of survival during prolonged anoxia.

Locomotory function under acute hypoxia

Anoxic coma onset data presented here suggests that an inhibition in the PKG signaling cascade promotes increased behavioral tolerance to acute low-oxygen environments before anoxic coma occurs (Fig. 1). Further, the data suggest that this pathway also acts through PDEs and PP2A (Fig. 2). We propose that the natural polymorphism in the *foraging* gene is functionally relevant to the limits for low-oxygen stress tolerance in fly behavior. Thus, oxygen tolerance may have played a pivotal role in how the rover and sitter alleles were selected for during the evolution of this polymorphism. Further, because rovers and sitters differ in their ability to tolerate low-oxygen stress, ecological implications of habitat limitations and sudden environmental changes may contribute to changes in allelic frequencies in the wild. One example of low oxygen stress on fruit flies is drowning due to excessive rainfall, a variable environmental factor in any habitat. The *foraging* gene functions in food-related behaviors across diverse taxa (Reaume and Sokolowski, 2009). Whether *for*'s function in oxygen tolerance is also conserved remains to be determined. Interestingly, previous work has shown that rover and sitter larvae differ in their food-related behaviors in response to low oxygen, where rover larvae leave food more quickly than sitters during mild hypoxia (Wingrove and O'Farrell, 1999).

Our results identify a molecular pathway involved in the modulation of anoxic coma – a response to acute hypoxia. This work also raises the possibility that future studies may reveal polymorphisms in genes encoding molecular targets described here affecting risk to low-oxygen stress pathologies. Further, the finding that molecules important for regulating the tolerance to acute hypoxia also function similarly during hyperthermic stress (Dawson-Scully et al., 2007) suggests that a conserved mechanism subserves both types of stress. At the level of the nervous system, these types of stresses may act to deplete cellular energy, due to hyperactivity during hyperthermia or blockage of cellular metabolism during hypoxia (Carling, 2004). At the cellular level, the reduction of whole-cell K^+ current may be a form of cellular energy conservation (Weckstrom and Laughlin, 1995) that confers tolerance to such stresses (Ramirez et al., 1999).

Animal survival under prolonged anoxia

Recent published work in non-anoxia-tolerant systems has demonstrated loosely that PKG activation is protective for cell survival during anoxia in mammals (Caretti et al., 2008). However, little is known about how this protection relates to the tolerance of neural function, and what components of the PKG pathway are involved. Through the use of the fruit fly, an anoxia-tolerant organism, we have demonstrated that the cGMP–PKG–PP2A pathway alters behavioral tolerance to acute hypoxia and mediates neuroprotection (a term that now includes neural function and survival). In the future it will be important to examine both

upstream modulators, to determine if adenosine (AD) signaling is a common link to PKG in anoxia tolerance, and downstream effectors to determine the effects of PKG on cell function and survival. The role of PKG in K_{ATP} channel regulation remains controversial, with some studies suggesting that PKG activates K_{ATP} (Han et al., 2001) while others report K_{ATP} suppression by guanyl cyclase (GC) (Ropero et al., 1999). Recent patch clamp work by Chai and Lin et al. (Chai and Lin et al., 2008) confirms that stimulation of the PKG pathway increases K_{ATP} activity, and the authors suggest that this is a means to manipulate neuronal excitability and/or survival (Chai and Lin, 2008). This correlates well with the results shown here using DCA, where flies have similar coma onset to DCA application as they do with PKG activation (Fig. 2A). Since previous work has shown that DCA increases whole-cell K^+ current (Michelakis et al., 2002; Michelakis et al., 2003; Bonnet et al., 2007), and that PKG activation leads to an increase in whole-cell K^+ current (Renger et al., 1999), we expected to see similar phenotypes from the addition of each. Another interesting finding was that DCA did not affect PKG activity levels whatsoever (Fig. 2B). Therefore, it appears that the K^+ current increase from DCA exposure may be direct or through another pathway other than the PKG pathway. Future work is required to determine which specific K^+ channels are affected by the manipulation of this pathway, and specifically which cellular or sub-cellular structure (plasma membrane *vs* mitochondria) these are found. In flies, the mammalian homolog of the SUR2 subunit of mK_{ATP} (dSUR) was also shown to play a protective role against hypoxic stress (Akasaka et al., 2006).

Function *vs* survival in the PKG pathway

The data here, then, suggest that inhibition of the PKG/PP2A pathway (Fig. 1) extends neural function in the face of acute hypoxia. By contrast, an up-regulation of the PKG pathway increases cell survival; it is not yet known if this occurs by a more rapid suppression of function, such as what is shown in turtles (Milton et al., 2002). Under anoxic stress, anoxia-tolerant animals such as turtles suppress metabolic requirements and enter a coma-like state. Therefore, it would be of interest to determine if fly coma onset is such a suppression. Our data suggest that the PKG pathway shows an inverse relationship between preserving function but inducing cell death during anoxia. Therefore, activating the PKG pathway confers protection of survival but reduces tolerance of neural function to acute hypoxia. Our current hypothesis is: inhibition of the PKG pathway leads to the protection of neural function during acute hypoxia, allowing for the nervous system to continue operating in the face of increased physiological stress. However, this protection comes at the cost of decreased survival, i.e. when the suppression of cell function is blocked, as with reduced PKG activity, mortality increases significantly. It has been suggested (for a review see Downey et al., 2008) that AD acts in part through the PKG pathway (Downey et al., 2008), so it is of interest to note that the blockade of AD receptors in the turtle *in vivo* significantly delays the onset of anoxic EEG suppression (Fernandes et al., 1997), a component that requires further study. To elucidate these other pathways of interest for animal survival during chronic anoxia we are currently attempting to develop a reliable pharmacological assay that will last multiple hours.

Because our work demonstrates that the PKG pathway can be manipulated to protect either function or survival, there is the potential to rapidly intervene with a pharmacological treatment to differentially protect either function or survival potentially at the cellular level, depending on what is required during a stroke event. However, the cellular mechanisms involved in both of these types of protection are still poorly understood, especially in their potential relationship with the mitochondria and the apoptotic pathway.

LIST OF ABBREVIATIONS

AD	adenosine
ANOVA	analysis of variance
DCA	dichloroacetate
DMSO	dimethyl sulfoxide
GC	guanyl cyclase
PDE5/6	phosphodiesterase
PKG	cGMP-dependent protein kinase
PP2A	protein phosphatase 2A

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