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Research Report

Inhibition of protein kinase G activity protects neonatal mouse respiratory network from hyperthermic and hypoxic stress

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ABSTRACT

In spite of considerable research attention focused on clarifying the mechanisms by which the mammalian respiratory rhythm is generated, little attention has been given to examining how this neuronal circuit can be protected from heat stress. Hyperthermia has a profound effect on neuronal circuits including the circuit that generates breathing in mammals. As temperature of the brainstem increases, respiratory frequency concomitantly rises. If temperature continues to increase respiratory arrest (apnea) and death can occur. Previous research has implicated protein kinase G (PKG) activity in regulating neuronal thermosensitivity of neuronal circuits in invertebrates. Here we examine if pharmacological manipulation of PKG activity in a brainstem slice preparation could alter the thermosensitivity of the fictive neonatal mouse respiratory rhythm. We report a striking effect following alteration of PKG activity in the brainstem such that slices treated with the PKG inhibitor KT5823 recovered fictive respiratory rhythm generation significantly faster than control slices and slices treated with a PKG activator (8-Br-cGMP). Furthermore, slices treated with 8-Br-cGMP arrested fictive respiration at a significantly lower temperature than all other treatment groups. In a separate set of experiments we examined if altered PKG activity could regulate the response of slices to hypoxia by altering the protective switch to fictive gasping. Slices treated with 8-Br-cGMP did not switch to the fictive gasp-like pattern following exposure to hypoxia whereas slices treated with KT5823 did display fictive gasping. We propose that PKG activity inversely regulates the amount of stress the neonatal mammalian respiratory rhythm can endure.

1. Introduction

The mammalian respiratory rhythm is generated by a population of neurons in the rostral ventrolateral medulla in a discrete region known as the ventral respiratory group (VRG). The VRG contains the kernel for the generation of the inspiratory rhythm termed the pre-Bötzinger complex (PreBötC; Smith et al., 1991). When isolated, slices containing this region are able to generate fictive respiratory rhythms in phase with motor output via the hypoglossal nerve (Peña et al., 2008). As with other central circuits, function derived from these rhythmically active neurons is directly affected by changes in

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temperature (Peever et al., 1999; Tryba and Ramirez, 2003), and is impaired if exposed to hyperthermic conditions (Tryba and Ramirez, 2004). Despite recent advancements in our understanding of how breathing behavior is coordinated (for reviews see: Rekling and Feldman, 1998; Feldman et al., 2003; Feldman and Del Negro, 2006; Doi and Ramirez, 2008) little attention has been given to exploring how this neuronal circuit can be protected from hyperthermic stress.

Previous research using *Drosophila* and locusts (*Locusta migratoria*), both established models for investigating CNS thermotolerance, suggest that decreased protein kinase G (PKG) activity increases thermotolerance of neuronal circuits coordinating feeding, locomotion, and ventilation (Dawson-Scully et al., 2007). In *Drosophila* two naturally occurring variants of the *foraging* (*for*) gene exist (Osborne et al., 1997). Rovers (for^R) have higher PKG transcript levels and move more in the presence of food than sitters (for^S), which have reduced PKG transcript levels. These variants have altered neuronal thermotolerance, such that pharmacologically or genetically reducing levels of PKG activity increases synaptic thermotolerance across the larval neuromuscular junction (Dawson-Scully et al., 2007). During a thermotolerance test for^S failed at significantly higher temperature than for^R. In addition, these variants display altered behavioral responses to hypoxia (Wingrove and O'Farrell, 1999). for^R appear to be more sensitive to hypoxia and actively locomote away from areas of low oxygen whereas for^S do not. Similarly, thermotolerance can be conferred to the neuronal circuit that coordinates ventilation in locusts following pharmacological inhibition of PKG activity (Dawson-Scully et al., 2007). During a thermotolerance test locusts treated with a PKG inhibitor could withstand significantly higher temperatures than animals treated with agonists of PKG (47 °C vs. 36 °C) and recovered more rapidly following hyperthermia-induced cessation of ventilation (58 vs. 155 s). Thus we reasoned that because the PKG signaling pathway is conserved between insects and mammals the neonatal respiratory central pattern generator (CPG) might be protected from hyperthermic stress following pharmacological inhibition of PKG activity. Given that high PKG levels are correlated with increased sensitivity to hypoxia in *Drosophila* (Wingrove and O'Farrell, 1999), we also tested the role of PKG signaling in the hypoxic response of the respiratory CPG. It is well known that the PreBötC undergoes a reconfiguration process that leads to fictive gasping generation *in vitro* (Lieske et al., 2000; Peña et al., 2004), however the intracellular signaling pathways that might modulate such reconfiguration remain unknown.

2. Results

2.1. Response to thermotolerance test

To examine the effect of temperature on the neonatal respiratory rhythm we positioned suction electrodes on the VRG and monitored population activity of neurons responsible for generating breathing behavior (Fig. 1A, B). Gradually raising artificial cerebrospinal fluid (ACSF) temperature (5 °C/min) significantly increased the frequency of the fictive rhythm from 0.29±0.06 Hz at 30 °C to 0.62±0.04 Hz at 35 °C in untreated slices ($n=13$; paired t-test, $df=12$, $P<0.001$). The VRG failed to generate a rhythm at 37.25±0.52 °C ($n=10$, Fig. 1C). Following heat-induced rhythm failure the heater was switched off allowing ACSF temperature to return to 30 °C. All slices recovered fictive rhythm generation. The length of time it took control slices to recover fictive respiration was 113.09±17.58 s. Rhythm frequency 1 min following recovery (0.25±0.04 Hz) and 20 min (0.27±0.03 Hz) returned to initial values (one-way ANOVA, $df=2$, $F=0.309$, $P<0.736$).

2.2. PKG inhibition increases rhythm thermotolerance

To investigate if pharmacological manipulation of the PKG signaling pathway could alter the thermosensitivity of this rhythm we utilized a within slice experimental design to compare various drug treatments. We quantified changes in rhythm failure temperature and recovery time. All treatment groups first received a thermotolerance test under control conditions (no drugs). After this, slices were allowed 10 min of recovery in ACSF before being treated with a 10 min bath-application of either 10 μM 8-Br-cGMP (PKG agonist) or 3 or 10 μM KT5823 (PKG activator). Following drug treatment a second thermotolerance test was performed. Raw values for failure temperature and recovery time are presented in Table 1. One slice treated with 8-Br-cGMP did not recover following the second thermotolerance test. As expected, we found no difference in failure temperature between the four groups following the first thermotolerance test (one-way ANOVA, $df=3$, $F=1.358$, $P=0.274$) nor did we find any differences in the recovery time (Kruskal–Wallis ANOVA on ranks, $df=3$, $H=4.389$, $P=0.222$). However, we did find changes following the second thermotolerance test. To account for slight variation between the groups we normalized differences between the first and second failure temperatures and recovery times respectively. We found significant differences between the four treatment groups (one-way ANOVA, $df=3$, $F=3.723$, $P=0.022$). To localize where the differences lay we used a post-hoc Tukey test and found a significant reduction in the failure temperature of 8-Br-cGMP-treated slices compared to the control group (Tukey test, $P=0.032$). No differences were found in slices treated with either concentration of KT5823 (Fig. 1D). However we did find significant changes in recovery times (one-way ANOVA, $df=3$, $F=7.827$, $P<0.001$). Slices treated with 10 μM KT5823 recovered circuit function significantly faster than control and 8-Br-cGMP-treated slices (Tukey test vs. control, $P=0.022$; vs. 8-Br-cGMP $P=0.002$). Moreover, slices treated with 3 μM KT5823 also recovered significantly faster than control and 8-Br-cGMP-treated slices (Tukey test vs. control, $P=0.050$; vs. 8-Br-cGMP $P=0.004$). No differences in recovery time were found between slices treated with 8-Br-cGMP and control preparations.

2.3. Additional responses to drugs

To examine the effect 8-Br-cGMP and KT5823 had on the fictive neonatal rhythm we examined the frequency and the stability of the rhythm for 2 min following a 10 min drug application (Fig. 2). We found no significant differences in fictive rhythm frequency between all treatment groups (Fig. 2B). To examine the stability of the rhythm we examined

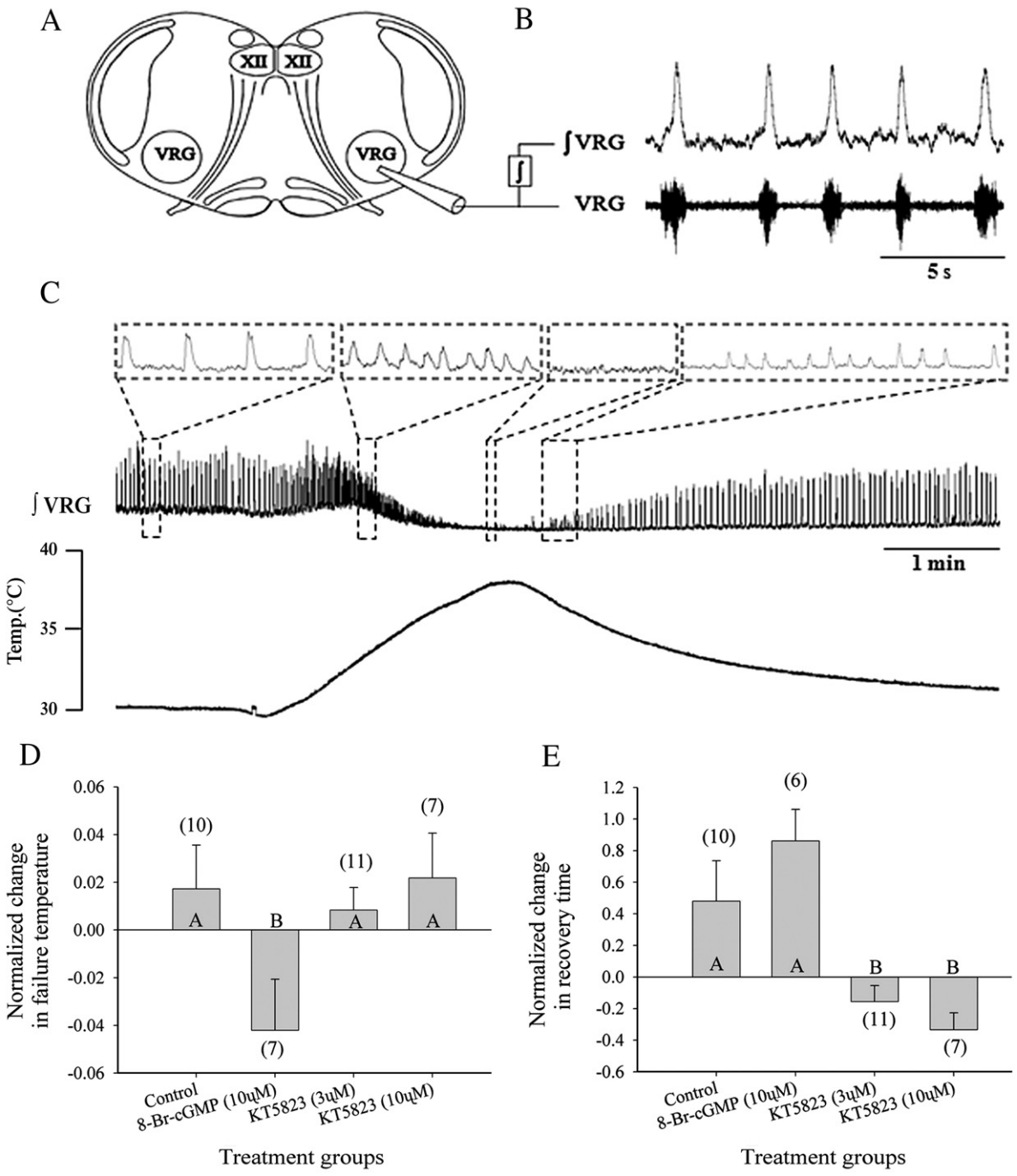

Fig. 1 – Sensitivity of the respiratory rhythm to temperature change is decreased following PKG inhibition. (A) Neonatal brainstem slice recordings of inspiratory bursting activity were recorded using a suction electrode placed on the surface of the slice near the ventral respiratory group (VRG). (B) Raw signals (VRG) were integrated (∫VRG). (C) Artificial cerebrospinal fluid (ACSF), held at 30 °C, was perfused over the medullary slice for 30 min; allowing the inspiratory rhythm to stabilize. Following this, ACSF temperature was raised (5 °C/min) until rhythm failure occurred (temperature ramp), at which point ACSF temperature was returned to 30 °C. A second temperature ramp was performed 30 min (last 10 min of this time period drugs were treated to the slice) after recovery from the first temperature ramp for within animal comparisons of drug treatments. (D) To examine the effect of drug treatment on the fictive rhythm failure temperature we normalized the second failure temperature (slices had been drug treated) to the first failure temperature (had not been treated yet). Brainstem slice preparations treated with ACSF containing 8-Br-cGMP (10 μM), a PKG agonist, displayed a significant reduction in the failure temperature compared to all other treatment groups. Inhibition of PKG activity with KT5823 had no effect. (E) Fictive rhythm recovery time was faster (shorter recovery time) in slices treated with both 3 and 10 μM KT5823. Slices treated with the activator did not take significantly longer to recover than control. Numbers in brackets represent sample sizes. Letters represent statistical groupings using a post-hoc test, whereby bars with different letters are significantly different and bars that share a letter are not (one-way ANOVA, Tukey, $p<0.05$).

Table 1 – Failure temperatures and recovery times of fictive respiratory pattern generation.

Treatment	First thermotolerance test		Second thermotolerance test	
	Failure temperature	Recovery time	Failure temperature	Recovery time
Control	37.25±0.52 °C	113.09±17.58 s	37.87±0.73 °C	156.08±28.62 s
10 μM 8-Br-cGMP	38.10±0.58 °C	117.49±15.88 s	36.45±0.54 °C	218.22±20.58 s
3 μM KT5823	39.01±0.84 °C	232.84±51.01 s	39.33±0.85 °C	185.63±38.94 s
10 μM KT5823	37.66±0.68 °C	127.06±0.68 s	38.45±0.75 °C	74.66±9.86 s

rhythm irregularity (Fig. 2A RI; see Peña and Ramirez, 2002, for explanation) scores during the same 2 min period (Fig. 2C). We found significant differences between RI scores (one-way ANOVA, $df=3$, $F=9.077$, $P<0.001$). To localize where the differences lay we ran an all pairwise multiple comparison procedure and found that slices treated with 8-Br-cGMP were significantly less stable than slices in all other treatments (Tukey Test, $P<0.008$; Fig. 2C).

2.4. *Response to hypoxia*

The fictive rhythm response to acute hypoxia was examined for 120 s after 8 min of exposure to 95% N_2, 5% CO_2 gas. As previously reported (Lieske et al., 2000; Peña et al., 2004, 2008), we found a significant change in the shape of each integrated burst after prolonged hypoxia, such change is associated with the transition from fictive eupnea generation in normoxia to fictive gasping generation in hypoxia (Lieske et al., 2000; Peña et al., 2004, 2008). Following exposure to hypoxia, fictive inspiratory burst shape changed from a bell shape (Fig. 3A, left) to a decrementing gasp-like shape (Fig. 3A, right). To quantify this change we calculated the rise time from 20% to 80% of fictive burst amplitude during eupnea. We found no change in the rise time between the four treatment groups during eupnea. However we did find that during hypoxia all treatments, except slices treated with 8-Br-cGMP, changed inspiratory burst

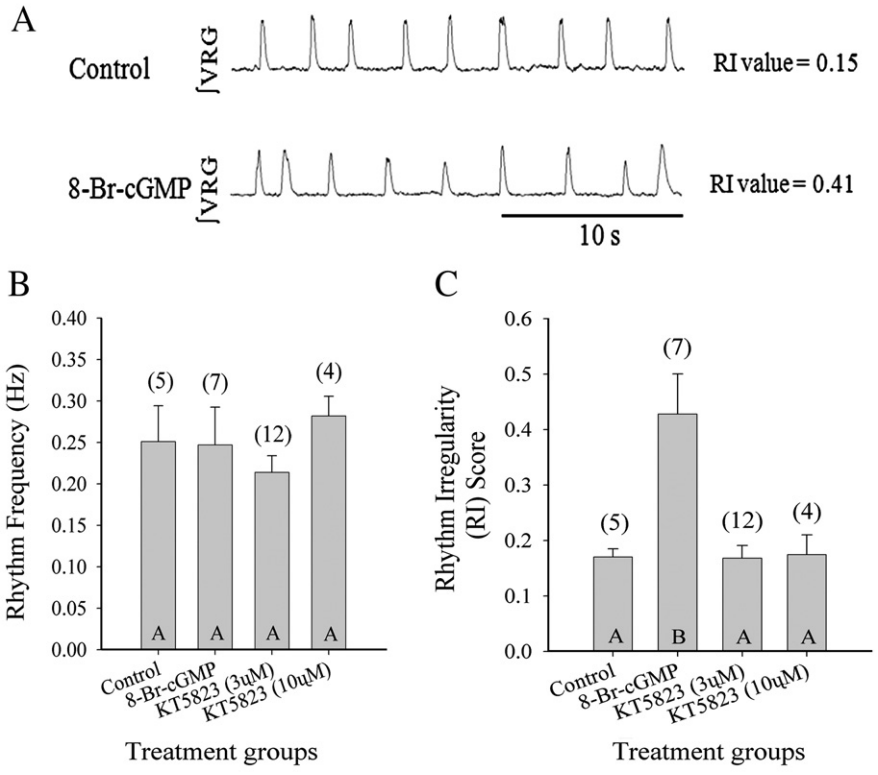

Fig. 2 – Fictive inspiratory neuronal activity recorded from the ventral respiratory group (VRG) from a mouse medullary brainstem slice is sensitive to PKG activation. (A) Sample traces of fictive inspiratory bursting activity following a 10 min bath application of artificial cerebrospinal fluid (ACSF) at 30°C, recorded using a suction electrode and integrated (∫EMG) (top) were more stable than preparations treated with a 10 min bath application of 8-Br-cGMP (10 μM) (bottom). (B) Rhythm frequency (Hz) of slices treated with 8-Br-cGMP did not differ between control slices or those treated with 3 or 10 μM KT5823. (C) However rhythm irregularity (RI) scores of animals treated with 10 μM 8-Br-cGMP were significantly greater than control animals and animals treated with 3 or 10 μM KT5823. Suggesting that as PKG activity increases in the VRG, stable rhythm generation is partially compromised. Numbers in brackets represent sample sizes. Letters represent statistical groupings using a post-hoc test, whereby bars with different letters are significantly different and bars that share a letter are not (one-way ANOVA, Tukey, $p<0.008$).

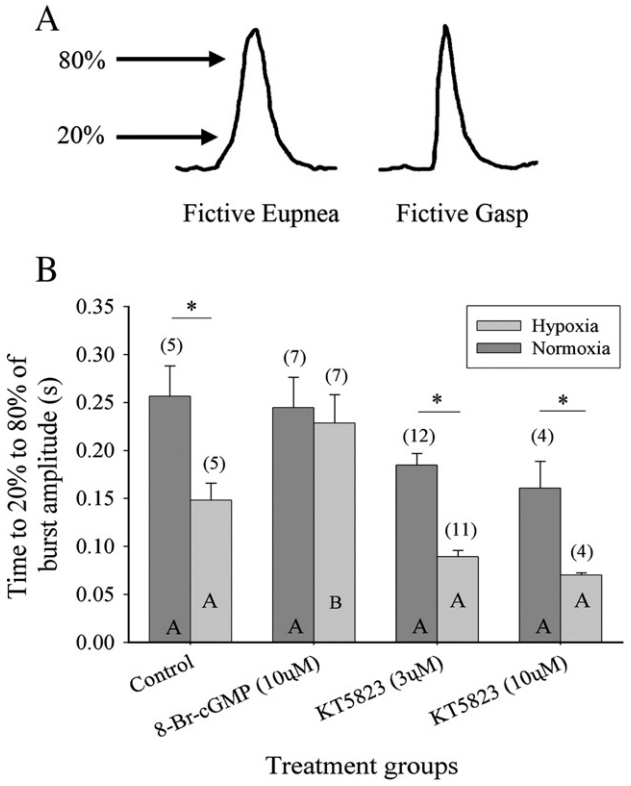

Fig. 3 – Fictive gasping behavior is absent during acute hypoxia in brainstem slices treated with 8-Br-cGMP. (A) Following exposure to hypoxia, fictive inspiratory burst shape changes from bell-shaped (left) to a decrementing gasp-like shape (right). It is believed that gasp-like behavior is protective because it promotes inspiratory effort and plays a role in autoresuscitation (see text). (B) To quantify differences in fictive inspiratory bursting patterns during exposure to hypoxia (perfusion of nonoxygenated ACSF) we calculated the rise time from 20% to 80% of fictive burst amplitude. Control slices showed a stereotyped normal gasp-like response to hypoxia, this was also observed in slices treated with 3 and 10 μM KT5823. However, slices pretreated with 8-Br-cGMP did not produce fictive gasp-like behavior during exposure to hypoxia. No drug treatment altered the rise time of burst amplitude during eupnea. However, amplitude rise time was significantly shorter in slices treated with 3 or 10 μM KT5823, and control preparations than slices treated with 10 μM 8-Br-cGMP. Numbers in brackets represent sample sizes. Asterisks indicate differences between hypoxia and normoxia within each group. Letters represent statistical groupings (separated into hypoxia and normoxia statistical groupings) using a post-hoc test, whereby bars with different letters are significantly different and bars that share a letter are not (two-way repeated measures ANOVA, with a Tukey multiple comparisons test, $p<0.05$).

pattern shape to the decrementing gasp-like shape (two-way repeated measures ANOVA $df=3$, $F=10.235$, $P<0.003$; Tukey test: control, $P=0.011$; 10 μM 8-Br-cGMP, $P=0.372$; 3 μM KT5823, $P=0.0.30$; 10 μM KT5823, $P=0.022$; Fig. 3B). Slices treated with 3 and 10 μM KT5823 did not have exaggerated gasp-like burst shapes compared to the control group.

3. Discussions

Genetic and pharmacological reductions in PKG activity in *Drosophila* increase thermotolerance of neuronal function and a comparable effect is observed in *L. migratoria* (Dawson-Scully et al., 2007). We monitored fictive rhythmic activity from the neonatal mouse VRG during a thermotolerance test and examined if altered PKG activity resulted in changes in brainstem slice tolerance to temperature stress. All experiments began with slices incubated (below the normal body temperature) at 30 °C and circulating ACSF temperature was raised in a ramp-like fashion (5 °C/min) until rhythm failure occurred (36–40 °C). We found a salient effect of PKG manipulation on the thermosensitivity of the neuronal network, such that brainstem slices treated with an activator of this pathway subsequently displayed decreased thermotolerance. Conversely, application of an antagonist reduced the time to recover fictive rhythm activity following heat-induced failure of circuit function which we equate to an increase in circuit thermotolerance.

Additionally, we examined if tolerance of the fictive rhythm to hypoxia could be altered by pharmacological manipulations of PKG activity. We found a striking effect of altering PKG activity, such that slices treated with the agonist did not switch to fictive gasping rhythm during the hypoxia test whereas slices treated with the PKG antagonist did display the protective fictive gasping pattern. Thus, we conclude that PKG plays an important role in regulating the tolerance of the neonatal mouse respiratory neuronal network to hyperthermia and hypoxia.

Within the CNS PKG activity is regulated by the nitric oxide/cyclic guanosine monophosphate/protein kinase G (NO/cGMP/PKG) pathway (for reviews see: Moncada et al., 1991; Guix et al., 2005; Yetik-Anacak and Catravas, 2006; Garthwaite, 2008). This pathway is highly conserved across disparate phyla. Increased PKG activity in the nervous system results from the production of nitric oxide (NO) in the same cell or nearby cells by the enzyme nitric oxide synthase (NOS). Three mammalian isoforms of NOS exist (neuronal NOS (nNOS); inducible NOS (iNOS); and endothelial NOS (eNOS)) all of which are well characterized. NO readily diffuses across cell membranes and interacts and increases the activity of soluble guanylyl cyclase which raises the $[cGMP]_i$ by catalyzing the conversion of GTP to cGMP. This increases the likelihood of cGMP binding to the regulatory domains on PKG thereby increasing kinase activity.

The role NO and its 2nd messenger pathway play in regulating the neuronal control of breathing behavior has been reviewed (Reeves et al., 2008). In bullfrogs (*Rana catesbeiana*) sodium nitroprusside (an NO donor) and L-arginine increase fictive respiratory frequency (Hedrick et al., 1998) and a similar observation has been reported in rat respiratory neurons (Pierrefiche et al., 2007). Increased activity from an array of brainstem nuclei results from NO neuromodulation. These include: nucleus of the solitary tract, the dorsal motor nucleus of vagus, the locus coeruleus, the rostral ventrolateral medulla, and the pontine respiratory group (Tagawa et al., 1994; Travagli and Gillis, 1994; Fabris et al., 2000; De Paula and Bronco, 2003; Ling et al., 1992). It seems likely that the excitatory effect of NO is through altered cGMP or PKG activities.

Although other derivatives of NO such as S-nitrosothiol and S-nitrosoglutathione have been implicated in altering respiratory activity (Jia et al., 1996; Lipton et al 2001; Gaston et al 2006), nonetheless altered concentrations of cGMP and PKG activity appear to play a large role in regulating breathing behavior. PKG is present throughout the nervous systems of mammals including nuclei involved in respiration (El-Husseini et al., 1995, 1999). Taken together with data from comparative animal models we reasoned that altered PKG activity might be involved in generating fictive respiratory rhythm thermotolerance.

In *L. migratoria* the upper temperature limit for continued ventilatory function is extended by pharmacologically decreasing PKG activity (Dawson-Scully et al., 2007). A core feature of hyperthermic neuronal failure in *L. migratoria* and in mice is the onset of a spreading depression-like event (SD) which exactly coincides with the moment of temperature induced-arrest of neuronal function (Wu and Fisher, 2000; Robertson, 2004; Rodgers et al., 2007). SD is characterized by a near complete depolarization of a majority of neurons that propagates at 2–5 mm/min in gray matter, although in invertebrates the depolarization is to a lesser extent with the propagation speed being at a similar velocity (Somjen, 2001; Rodgers et al., 2007; Armstrong et al., 2009). The depolarization is due to a failure of ionic homeostatic regulation which leads to a substantial redistribution of ionic concentrations of potassium, sodium, chloride and calcium. More specifically, tightly controlled baseline levels of 3.0–3.5 mM extracellular potassium concentration ($[K^+]_o$) rapidly rise to 50.0–60.0 mM $[K^+]_o$ during SD in the rat hippocampus (Vyskočil et al., 1972; Müller and Somjen, 2000). A similar observation has been made in the locust metathoracic ganglion where baseline levels of 8–12 mM extracellular potassium concentration ($[K^+]_o$) rapidly rise to ~ 52.0 mM $[K^+]_o$ during SD-like events (Rodgers et al., 2007). Preconditioning treatments which extend the operational temperature range of neuronal circuits during heat stress do so by delaying the onset of SD (Rodgers et al., 2007). Furthermore, the onset of SD-like events in locust is attenuated following inhibition of PKG activity (Armstrong et al., 2009). This results in an increase in neuronal thermotolerance observed in *L. migratoria* and possibly in *Drosophila* for^S mutants as well (Dawson-Scully et al., 2007). Although SD was not investigated here, it is possible that a heat-induced SD-like event occurs in the VRG during hyperthermia and accounts for the arrest of fictive respiration observed in our slices. SD does occur in the brainstem and spinal cord (Takahashi et al., 1981; Czéh and Somjen, 1990; Streit et al., 1995; Richter et al., 2003) however these regions have been noted for being more resistant to its occurrence possibly due to the higher proportion of white matter in adult animals (Somjen, 2001). Recently, Funke et al. (2009) showed that during severe hypoxia the VRG, hypoglossal nucleus and the nucleus of the solitary tract of the neonatal rat undergo hypoxia-induced SD. Thus the likelihood exists that hyperthermia-induced fictive respiratory arrests coincide with a SD-like event in the brainstem.

A proposed mechanism for delaying the onset of a SD-like event and the concomitant cessation of network function could result from treatments that decrease $[K^+]_o$ build up during stress (Robertson, 2004; Armstrong and Robertson, 2006). One way in which this could be accomplished is via attenuating K^+ conductances. This occurs following preconditioning treatments in locusts (Ramirez et al., 1999, Wu et al., 2002) and is believed to account for thermoprotection conferred following treatment with TEA, serotonin or octopamine (Wu et al., 2001; Newman et al., 2003; Armstrong et al., 2006). Interestingly, reduced neuronal steady-state K^+ currents are hallmark features of the for^S mutants (Renger et al., 1999). Along with attenuated K^+ conductances, improved K^+ clearance via increased Na^+/K^+ATPase activity has also been postulated to maintain ionic homeostasis during hyperthermia (Robertson, 2004; Armstrong and Robertson, 2006). Taken together there is a mounting body of evidence which suggests that thermoprotection of circuit function is in part derived from mechanisms which decrease the build up of $[K^+]_o$ and those that increase the clearance rates of K^+ from the extracellular space during stress. Activation of K^+ conductances has been demonstrated in mammalian tissues following activation of the NO/cGMP/PKG signaling pathway (Kubokawa et al., 1998; Cuong et al., 2006). In the mammalian basal forebrain and in the locus coeruleus this pathway activates a long lasting leak K^+ current leading to neuronal hyperpolarization (Xu et al., 1998; Kang et al., 2007). This hyperpolarization is dependent upon E_k being large; however during hypoxia and hyperthermia E_k may be reduced, thus NO stimulation could lead to a depolarization and predisposing the tissue toward generating a SD-like event (Armstrong et al., 2009).

We also examined if tolerance to hypoxia was affected by altered PKG activity. In 5 day old rat pups exposure to hypoxia results in a four discrete time periods. These are characterized by an initial increase in respiratory frequency, followed by primary apnea and then gasping and lastly terminal apnea (for review see: Feweel, 2005). A similar effect is observed in 5 to 10 day old mice pups (Tryba et al., 2006; Peña et al., 2008) our experimental mice were aged 5 to 6 days. As expected from previous work (Tryba et al., 2006; Peña et al., 2008) after 8 min of hypoxia fictive inspiratory burst shape changed from a bell shape to a decrementing gasp-like shape (Fig. 3A). It is believed that this switch in the activity pattern is protective because it acts as a mechanism for autoresuscitation, the failure of which may underlie sudden infant death syndrome (Sridhar et al., 2003). In this study we found that slices treated with 8-Br-cGMP did not produce fictive gasping during hypoxia (Fig. 3B). Conversely, slices treated with the PKG antagonist produced the strongest fictive gasp pattern by having the shortest time from 20% to 80% of burst amplitude (Fig. 3B). Interestingly, NO appears to play a crucial role in the ventilatory response to hypoxia, whereby hypoxia increases the activity of the NO-cGMP pathway in the brainstem (Gozal and Torres, 2001) and delays the beginning of hypoxic ventilatory depression (Haxhiu et al., 1995). This may account for our observations which showed that slices treated with the PKG agonist did not switch to the fictive gasp-like pattern following 8 min of hypoxia. Although it was not tested, it is conceivable that longer exposures of hypoxia to slices treated with the PKG agonist might eventually display fictive gasp-like patterns. Arguments for continued maintenance of fictive eupnea despite being exposed to acute hypoxia are difficult to make. It is possible that increased PKG activity coordinates other aspects of ventilatory changes during hypoxia which are

essential, such as maintained phrenic nerve and diaphragm activity (Haxhiu et al., 1995).

In summary, the results of this study suggest that impairment of the VRG due to hyperthermia and acute hypoxia can be mitigated by inhibition of PKG activity and exacerbated by its activation in the brainstem.

4. Experimental procedures

All experiments were approved by the Local Committee of Ethics on Animal Experimentation, which followed the regulations established in the Mexican Official Norm for the Use and Care of Laboratory Animals ('Norma Official Mexicana' NOM-062-ZOO-1999) and the Ethical Committee on Centro de Investigación y Estudios Avanzados.

4.1. *Slice preparation*

Details for the preparation of the brainstem slice have been previously reported (Peña et al., 2008). CD-1 outbred mice (Charles River Laboratories, Wilmington, MA) aged 5 or 6 days were quickly decapitated under anesthesia and the brain and brainstem were placed in ice-cooled artificial cerebrospinal fluid (ACSF) aerated with carbogen (95% O_2 and 5% CO_2). The ACSF was composed of (in mM): 119 NaCl, 3 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, and 30 D-glucose (pH 7.4). The brainstem was removed from the brain and glued rostral side up onto an agar block mounted into a vibratome (The Vibratome Company). The brainstem was serially sliced until the appearance of the inferior olive at which point a single 700 μm thick slice was produced and transferred to a recording chamber. The recording chamber was constantly superfused with aerated ACSF held at 30°C. KCl was gradually added to the ACSF solution raising the concentration from 3 to 8 mM over a span of 30 min before recordings were made.

4.2. *Thermotolerance test*

Suction electrodes were placed on the VRG to monitor fictive respiratory activity (Fig. 1A). The signal was filtered (high pass cutoff, 5 Hz; low pass 0.25 kHz cutoff) and then amplified with a wide-band AC amplifier (Grass Instruments). Following this, the signal was rectified and integrated using an electronic filter (time constant of 30–50 ms; see Peña et al, 2008). ACSF temperature was carefully controlled during the experiments, which began by monitoring the fictive bursting pattern for 10 min. Following this we gradually increased in a ramp-like manner (5°C/min) the temperature of the ACSF until rhythm failure occurred thereupon the temperature was allowed to return to 30 °C facilitating recovery of spontaneous rhythm generation. The temperature at which rhythm generation failed and the length of time taken to recover were measured. A second hyperthermic challenge was performed 20 min following the first hyperthermic challenge. However, 10 min prior to the challenge ACSF containing 10 μM 8-Br-cGMP (PKG agonist) or 10 or 3 μM of KT5823 (PKG antagonist) was used to bathe the slice. Slices designated as controls were continuously bathed in ACSF. All drugs were dissolved with a minimum amount of DMSO (0.05% by volume). Control preparations also used ACSF with DMSO. The second failure temperature and recovery time were compared to the first values to examine the effect of drug treatment.

4.3. *Acute hypoxia tolerance test*

In a second set of experiments rhythmic slices were placed under hypoxic conditions (95% O_2 and 5% CO_2 replaced with 95% N_2 and 5% CO_2) for 10 min. Changes in rhythm frequency, and the rise time of the integrated fictive respiratory activity trace were quantified following pharmacological manipulation of the PKG signaling pathway. Sections of 120 s before hypoxia treatment began and after 8 min in hypoxia of recorded data were used to quantify changes in the rhythm.

4.4. *Statistics*

SigmaStat 3.1 was used to assess data groupings for statistical significance. Statistical analyses used paired *t*-test, and one-way and two-way ANOVA, followed by a repeated measures post-hoc Tukey multiple comparison test. For non-parametric statistical tests we used the Mann–Whitney Rank Sum Test. For within slice comparison of failure temperature and recovery time we used a Kruskal–Wallis ANOVA on ranks. Figures show significant differences using letter designations, treatments containing same letters are not significantly different from one another. Significance was assessed at $P<0.05$ however the majority of P values were <0.001.

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