A cGMP-dependent protein kinase (PKG) controls synaptic transmission tolerance to acute oxidative stress at the \textit{Drosophila} larval neuromuscular junction

Stacee Lee Caplan, Sarah L. Milton, and Ken Dawson-Scully
Department of Biological Sciences, Florida Atlantic University, Boca Raton, Florida

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Caplan SL, Milton SL, Dawson-Scully K. A cGMP-dependent protein kinase (PKG) controls synaptic transmission tolerance to acute oxidative stress at the \textit{Drosophila} larval neuromuscular junction. \textit{J Neurophysiol} 109: 649–658, 2013. First published November 7, 2012; doi:10.1152/jn.00784.2011.—Increasing evidence demonstrates that modulating the cGMP-dependent protein kinase G (PKG) pathway produces an array of behavioral phenotypes in the fruit fly, \textit{Drosophila melanogaster}. Altering PKG activity, either genetically via the \textit{foraging} (\textit{for}) gene or using pharmacology modifies tolerance to acute abiotic stresses such as hyperthermia and hypoxia. PKG signaling has been shown to modulate neuroprotection in many experimental paradigms of acute brain trauma and chronic neurodegenerative diseases. However, relatively little is known about how this stress-induced neuroprotective mechanism affects neural communication. In this study, we investigated the role PKG activity has on synaptic transmission at the \textit{Drosophila} larval neuromuscular junction (NMJ) during acute oxidative stress and found that the application of 2.25 mM hydrogen peroxide (H$_2$O$_2$) disrupts synaptic function by rapidly increasing the rate of neuronal failure. Here, we report that reducing PKG activity through either natural genetic variation or an induced mutation of the \textit{for} gene increases synaptic tolerance during acute oxidative conditions. Furthermore, pharmacological manipulations revealed that neurotransmission is significantly extended during acute H$_2$O$_2$ exposure upon inhibition of the PKG pathway. Conversely, activation of this signaling cascade using either genetics or pharmacology significantly reduced the time until synaptic failure. Therefore, these findings suggest a potential role for PKG activity to regulate the tolerance of synaptic transmission during acute oxidative stress, where inhibition promotes functional protection while activation increases susceptibility to neurotransmission breakdown.

organism relies on a crucial intrinsic defense mechanism that involves the cGMP-dependent protein kinase G (PKG) pathway (Dawson-Scully et al. 2007, 2010). Analogous protection from modulating PKG signaling during acute hyperthermia and hypoxia has also been demonstrated in other species, such as the locust (Dawson-Scully et al. 2007), tadpole (Robertson and Sillar 2009), and mouse (Armstrong et al. 2010). Conservation of this pathway between insects and mammals implies its importance as a stress-mediated regulator that controls nervous system tolerance throughout severe cellular predicaments.

PKG activity is encoded by the \textit{Drosophila} foraging (\textit{for}) gene, a homolog of the human \textit{PRKG1} gene, and is expressed as a polymorphism that modulates behavioral food-searching strategies (Sokolowski 1980). Two variants of this gene exist in nature, where 70% of flies express the rover (\textit{forR}) allele with high PKG activity and 30% of flies express the sitter (\textit{forS}) allele with low PKG activity (Osborne et al. 1997). These natural fly populations display differential feeding behaviors, whererovers travel more than sitters in the presence of food, that remain throughout their entire life cycle (de Belle and Sokolowski 1987; Pereira and Sokolowski 1993). In addition to affecting foraging strategies, \textit{for} influences other functions, such as learning and memory (Mery et al. 2007) and acute stress tolerance (Dawson-Scully et al. 2007, 2010), as well as fundamental neuronal properties including excitability, transmitter release (Feil and Kleppisch 2008), synaptic plasticity (Kleppisch and Feil 2009), and potassium channel conductance (Renger et al. 1999).

In the present study, we analyzed synaptic transmission at the \textit{Drosophila} larval neuromuscular junction (NMJ) glutamatergic synapse during 2.25-mM H$_2$O$_2$ exposure. A combination of genetics and pharmacological approaches was used to explore the hypothesis that the PKG pathway is involved in controlling cellular responses to acute physiological stress by modulating neurotransmission. More specifically, we investigated if altering the PKG signaling cascade can regulate synaptic tolerance during acute oxidative stress and if this mechanism potentially occurs through downstream ion channel modification. PKG activity was manipulated in the following ways: 1) naturally using rover (\textit{forR}) or sitter (\textit{forS}) larvae, 2) genetic modification with \textit{forR} larvae that possess a mutation, and 3) via pharmacological disruption with the PKG inhibitors KT-5823 and Rp-β-phenyl-1,1′-ethanoguanosine 3′,5′-cyclic monophosphorothioate sodium salt hydrate (Rp-8-bromo-cGMP), the PKG activator 3′,5′-cyclic monophosphate 8-bromosodium salt (8-bromo-cGMP), and the K$^+$ channel inhibitor tetraethylammonium hydroxide (TEA).

UNLIKE MAMMALS, INSECTS POSSESS numerous strategies that enable survival during extreme ecological conditions. The fruit fly, \textit{Drosophila melanogaster}, is capable of handling drastic fluctuations in temperature and oxygen levels, allowing survival without causing serious damage to cells and tissues (Chen and Walker 1994; Feder 1997; Haddad 2006; Hoffmann et al. 2003; Misener et al. 2001; Wingrove and O’Farrell 1999). Since fruit flies are able to endure these conditions, they are an ideal model system for studying endogenous protective mechanisms that facilitate tolerance of the nervous system to acute stress. In response to experiencing drastic environmental changes in atmospheric temperature and oxygen levels, this
PKG ALTERS SYNAPTIC ACTIVITY DURING ACUTE H₂O₂ EXPOSURE

MATERIALS AND METHODS

Animals. Drosophila melanogaster third instar wandering larvae (~110-h old) from three wild-type strains, yellow-white (yw), rover (for⁸), and sitter (for¹) were used in this study. The for⁸ and for¹ strains are natural allelic variants of the Drosophila for gene. The mutant for⁸ strain was previously generated on a for¹ genetic background with a mutation in for that produces lower PKG activity/transcript levels than for¹ (Pereira and Sokolowski 1993) and was utilized to control for genetic background. All larvae were raised within an equal population density (~100 flies per bottle) on 50 ml of a standard Bloomington stock fly food in an incubator at 25°C under a 12:12-h light-dark light cycle.

Electrophysiology. Individual Drosophila larvae were collected and placed in a glass-dissecting dish containing 2 ml of Schneider’s insect medium (Sigma, St. Louis, MO). Each larva was positioned with the dorsal side up on the dissecting dish using standard insect pins. Removal of the internal organs and central nervous system was achieved by making a longitudinal cut in the anteroposterior direction along the dorsal surface to expose the underlying segmental muscles and nerves. An extracellular glass suction electrode was used to stimulate segmental nerves in muscle segments. The excitatory junction potential (EJP) was recorded from muscle 6 abdominal segments A3–4 with a sharp intracellular glass recording electrode filled with 3 M KAc (~40 MΩ).

The preparation medium was replaced with HL-3 saline (1.5 mM CaCl₂, 20 mM MgCl₂, 5 mM KCl, 70 mM NaCl, 10 mM NaHCO₃, 5 mM BES, 115 mM sucrose, 5 mM trehalose·2H₂O) made fresh daily (Macleod et al. 2002; Stewart et al. 1996). EJP recordings were viewed with an oscilloscope and digitally stored using the Scope program (AD Instruments, Colorado Springs, CO) for analysis. Evoked EJPs from repetitive stimulation (0.3-ms pulses delivered suprathreshold with a 1-Hz frequency) of both axons in larval muscle 6 were recorded in a stop-flow condition. EJP recordings were taken until synaptic transmission failure (amplitude <1 mV) occurred.

Intracellular recordings of the resting membrane potential (RMP) and input resistance were measured from larval muscle 6 with signals amplified by an IX1 intracellular preamplifier (Dagan, Minneapolis, MN). These measurements were taken as previously described (Zhang and Stewart 2010). Briefly, RMP measurements were taken from animals if the initial potential stabilized between ~60 to ~70 mV. If the membrane potential was equal to ~45 mV or more depolarized, the preparation was discarded. Input resistance was measured by injecting small current pulses of 2 nA (40-ms duration at 1-Hz frequency) applied continuously. Electrode resistance was canceled prior to measuring the input resistance by adjusting the bridge balance control. Resistance was calculated using Ohm’s law and only muscle fibers with an initial input resistance >5 or <40 MΩ were assayed.

Pharmacological manipulation. The same electrophysiology preparation assay described above was performed; however, animals were also exposed to acute oxidative stress and pharmacological agents that affect the PKG pathway. Acute oxidative stress was induced in larval preparations by adding 2.25 mM H₂O₂ to the HL-3 saline. Larvae subjected to H₂O₂ and simultaneous PKG drug treatment included one of the following agents dissolved in DMSO (all chemicals obtained from Sigma, St. Louis, MO): 40 μM 8-bromo-cGMP; (a PKG activator; Ruth et al. 1991), 1 μM KT-5823 (a selectively competitive, competitive PKG inhibitor; Grider 1993; Kase et al. 1987), or 50 μM Rp-8-bromo-cGMP (a more selective PKG inhibitor that irreversibly binds to the kinase; Petrov et al. 2008; Wei et al. 1996). These concentrations were previously shown to alter PKG activity in insect and mammalian models (Dawson-Scully et al. 2007; Li et al. 2007).

In a separate assay, pharmacological PKG agents were examined for adverse effects on the parameters of the EJP, where larvae were exposed to each drug separately and the EJP recordings were compared with controls for differences in amplitude, waveform/shape, and latency values. For drug combinations, larvae were exposed to acute oxidative stress (2.25 mM H₂O₂), a PKG agonist (40 μM 8-bromo-cGMP), and 250 μM TEA, a nonselective potassium (K⁺) channel inhibitor. A dose-response assay was performed to determine the concentration used. In the dose response assay it was found that 250 μM of TEA had no detrimental affect on the preparation, including no changes to the EJP. However, the preparation was compromised with increasing concentrations of TEA where 500 μM caused sporadic contractions ~15 min into the experiment, 1 mM caused sporadic contractions in ~5 min, and 2 mM caused contractions immediately upon application.

Statistics. All data were analyzed using SigmaStat (Chicago, Illinois). A one-way ANOVA test was used to compare significant differences across all treatment groups and the Holm-Sidak method to determine significance using multiple comparisons. Statistical significance (P < 0.05) was assigned using letter designations, where different letters show significant differences and the same letter assignments are not significant. The letter assignments begin with “A” representing the highest mean, “B” indicating the next highest, and so forth. All vertical bar charts are shown with the means ± SE.

RESULTS

Natural variation in PKG activity modulates synaptic tolerance to acute H₂O₂ exposure. To investigate if the PKG pathway is able to modulate synaptic transmission tolerance to acute oxidative stress, 2.25 mM H₂O₂ was exogenously applied to Drosophila larval NMJ preparations. Larvae of three different fly strains, for⁸, for¹, and for², with genetically different PKG activity levels were utilized to distinguish if any differences in neural communication during the acute stress existed and could be associated with differences in PKG activity. Wild-type for⁸ larvae express high PKG levels, wild-type for¹ larvae possess low PKG levels, and mutant for² larvae have a for² genetic background with a mutation in for that produces lower PKG levels comparable to for¹ larvae.

Under acute stress conditions, the PKG allelic variants demonstrated differential synaptic transmission tolerances indicated by varying rates across all treatment groups and the Holm-Sidak method to determine significance using multiple comparisons. Statistical significance (P < 0.05) was assigned using letter designations, where different letters show significant differences and the same letter assignments are not significant. The letter assignments begin with “A” representing the highest mean, “B” indicating the next highest, and so forth. All vertical bar charts are shown with the means ± SE.

Pharmacological agents do not disrupt EJP characteristics. The data indicated that genetically altered PKG expression modulates synaptic transmission tolerance to acute H₂O₂ exposure, but it did not demonstrate if wild-type yw Drosophila with chemically modulated PKG activity would exhibit similar differences in synaptic susceptibility to bath-applied H₂O₂ (2.25 mM). Before this question was addressed, we examined whether pharmacological PKG modulators would adversely affect parameters of the NMJ postsynaptic response (i.e., the EJP latency time, shape, amplitude). Pharmacological agents that either activate (+PKG, 8-bromo-cGMP) or inhibit (~PKG, KT-5823 and Rp-8-bromo-cGMP) PKG activity were...
PKG alters synaptic activity during acute H₂O₂ exposure. Therefore, any differences among evoked EJPs from yw larvae during the acute stress condition were attributed to altered PKG activity from chemical manipulation of the signaling cascade.

Chemical PKG manipulation alters synaptic function during acute oxidative stress. To demonstrate that the PKG pathway modulates synaptic transmission tolerance to acute oxidative stress, yw flies with chemically modified PKG activity were exposed to 2.25 mM H₂O₂. This H₂O₂ concentration was chosen to create a standard time frame to compare changes in NMJ synaptic failure times during pharmacological manipulation of PKG activity. Modification of the PKG pathway was achieved through pharmacological agents that directly activate (40 μM 8-bromo-cGMP) or inhibit (1 μM KT-5823 and 50 μM Rp-8-bromo-cGMP) PKG cellular function. When the EJP amplitude decay was plotted as a function of time, the differential drug treatments separate into clear patterns of varying synaptic transmission tolerance during the acute stress condition (Fig. 3A). Control preparations not exposed to acute oxidative stress nor any drug displayed a maximal time frame of 142 min before failure of synaptic transmission occurred in the stop-flow experimental assay. Activation of the PKG pathway during H₂O₂ exposure reduced the maximum time until synaptic breakdown from 52 to 44 min. Conversely, inhibition of the PKG pathway during acute H₂O₂-induced stress extended the maximum time until synaptic transmission failure from 52 to 67 or 84 min depending on the drug administered. These results suggest that during acute oxidative stress PKG inhibition extends the time until synaptic transmission failure, while PKG activation reduces this time frame.

Additionally, there were significant differences between the average synaptic failure times of all treatment groups (control, H₂O₂, H₂O₂/+PKG, and H₂O₂/−PKG) depending on the drug administered [one-way ANOVA, F(4,22) = 387.24, P < 0.001; Fig. 3B]. Animals exposed to 2.25 mM H₂O₂ displayed synaptic failure at an average of 46 ± 1 min compared with control preparations that occurred at an average of 131 ± 3 min. Elevated synaptic transmission tolerance during acute oxidative stress was observed in larvae treated with PKG inhibitors (−PKG). The addition of 1 μM KT-5823 or 50 μM Rp-8-bromo-cGMP increased the average synaptic failure time to 62 ± 3 and 79 ± 2 min, respectively. In contrast, animals treated with a PKG activator (40 μM 8-bromo-cGMP, +PKG) showed a reduction in the average time until synaptic transmission failure occurring at 39 ± 1 min. These results indicate that pharmacological manipulation of the PKG pathway can either protect or sensitize synaptic transmission tolerance during acute oxidative stress.

Modulating K⁺ channel kinetics affects neuromuscular transmission during acute stress. To explore potential mechanisms responsible for PKG’s effect on synaptic transmission during acute oxidative injury, we combined pharmacological manipulation of K⁺ channel kinetics and the PKG pathway. Individual wild-type yw larvae were exposed to acute oxidative stress (2.25 mM H₂O₂), a K⁺ channel inhibitor (250 μM TEA), and a PKG activator (40 μM 8-bromo-cGMP, +PKG), both individually and concurrently. Similar to the paradigm described in Fig. 2, we examined the EJP waveform before the application of the pharmacological compounds and 10-min postapplication and determined that there was no immediate change in the waveform caused by the compounds (Fig. 4). The drug combination revealed that widespread K⁺ channel inhi-
bition slowed the average decay rate of EJP amplitudes decline over time not only in the presence of H2O2 but also during PKG pathway activation (Fig. 5A). TEA extended the maximum time until synaptic transmission failure during the acute stress condition from 53 to 73 min. Additionally, this reduction in the EJP amplitude decline rate continued in the presence of the PKG agonist with the maximum failure time occurring at 70 min.

*Drosophila* NMJ synaptic transmission failure times of all treatment groups (H2O2, H2O2/TEA, and H2O2/TEA/H11001 PKG) were significantly altered by modifying K+ channel kinetics during acute H2O2 exposure [one-way ANOVA, F(2,15) = 31, P < 0.001; Fig. 5B]. Animals exposed to TEA and H2O2 exhibited an extended average synaptic transmission failure time occurring at 66 ± 2 min. This functional protection persisted during simultaneous PKG pathway activation with the average synaptic failure time occurring at 61 ± 2 min. These drug combinations suggest that reducing overall K+ conductance during acute oxidative stress protects synaptic function and that this protection overrides PKG manipulations, implicating these channels as potential downstream effectors of PKG activity in the signaling mechanism.

**Characterizing the mechanisms of synaptic failure from acute oxidative stress.** To determine if synaptic failure during acute oxidative stress occurs as a result of presynaptic or postsynaptic mechanisms, we measured the RMP and input resistance of the postsynaptic cell over a prolonged period of time (Fig. 6). This was done under three pharmacological conditions (H2O2, H2O2/+, and H2O2/−) in separate experiments. During the 2.25-mM H2O2 treatment, the resting membrane potential begins to depolarize at 30 min, and this time frame is altered by PKG manipulation (Fig. 6A). During PKG activation, the membrane depolarizes at a faster rate (~20 min), while PKG inhibition extends this time frame until ~60 min. The input resistance of the larval muscle approaches zero for all of these treatments; however, it does so at different rates of decline (Fig. 6B). PKG activation displays the steepest decay rate beginning at ~20 min, while this decline in resistance is delayed until ~30 min and ~50 min during H2O2 application and PKG inhibition, respectively.

**DISCUSSION**

The main findings from the present study show that PKG pathway manipulation, whether genetically or pharmacologi-
PKG alters synaptic activity during acute H$_2$O$_2$ exposure

**PKG signaling in the invertebrate nervous system.** Nitric oxide (NO) is a short-lived signaling molecule that readily diffuses across membranes and has been implicated as a neuromodulator in the central nervous system (Boehning and Snyder 2003; Garthwaite 2008; Snyder and Bredt 1991). NO is generated by NO synthases, and NO-sensitive guanylyl cyclases (NO-GCs) act as receptor molecules (Mergia et al. 2009). NO-GCs upregulate the amount of cGMP, which activate targets such as PKG (Hofmann et al. 2006). Properties of NO synthase have been characterized in numerous model organisms including *Drosophila* (Muller 1994). NO/cGMP signaling has been identified during postembryonic insect development at the fruit fly NMJ with NO most likely acting as the presynaptic retrograde messenger (Wildemann and Bicker 1999a,b), mirroring its organization in mammalian systems (Hopkins et al. 1996; Southam and Garthwaite 1993). Therefore, the larval NMJ provides a good model to study how synaptic transmission is modulated by PKG activity during acute oxidative stress.

**H$_2$O$_2$ exposure as a paradigm to study PKG signaling effects on neural function.** Organisms respond differently to cellular stress, and the basis for differences among species is still not fully understood. There are many different types of environmental stressors that cause cellular damage; however, the physiological effects on organisms converge on one common theme, the elevated generation of reactive oxygen species. Numerous stress events are associated with the increased intracellular production of reactive oxygen species, such as hyperthermia (Flanagan et al. 1998), hypoxia (Waypa et al. 2001), oxygen deprivation (Liu et al. 2005), and reperfusion following an ischemic event (Ferriero 2001).

Experimental use of H$_2$O$_2$ as a model for acute oxidative stress has been employed to imitate the pathology of numerous neurological disorders including cerebral ischemia (Methy et al. 2008; Wei et al. 2011), Amyotrophic lateral sclerosis (Nani et al. 2010), Parkinson’s disease (Jenner 2003), Friedreich’s ataxia (Anderson et al. 2008), and Alzheimer’s disease (Bhel et al. 1994; Miyata and Smith 1996). In addition to being a classic oxidative stress paradigm, H$_2$O$_2$ has been shown to modulate neuromuscular activity in vertebrates (Giniatullin and Giniatullin 2003) and manipulate neuronal signal transduction in mammalian cell culture (Chai and Lin 2010). This study exposed *Drosophila* larval NMJ preparations to 2.25 mM H$_2$O$_2$ and demonstrates that PKG manipulation alters synaptic transmission tolerance to acute oxidative toxicity. This H$_2$O$_2$ model provides a unique avenue for finding novel PKG signaling targets that may promote cell function and survival during acute stress insults.

**Genetic variation in PKG activity modulates synaptic function during H$_2$O$_2$ application.** We took advantage of the polymorphic expression of *for* to investigate if the PKG pathway would demonstrate similar command over acute oxidative stress tolerance as it has been shown to modulate neurological function and promote behavioral tolerance to other types of physiological stressors. Our data reveal that this signaling mechanism modulates synaptic function during H$_2$O$_2$-mediated cellular stress. Animals with inherently lower PKG activity (*for*<sup>6</sup>) maintain synaptic performance longer than those with higher PKG activity (*for*<sup>6</sup>) during acute stress exposure. The
Fig. 4. Tetraethylammonium hydroxide (TEA) at 250 μM does not affect distinctive EJP features. Representative traces of the evoked EJP response from yw Drosophila larval muscle 6 after differential drug treatments. Animals were bathed in 2 ml of HL-3 saline containing one of the following exposure conditions: control (saline only), 2.25 mM H$_2$O$_2$, 250 μM TEA, 250 μM TEA/2.25 mM H$_2$O$_2$, 250 μM TEA/2.25 mM H$_2$O$_2$/40 μM 8-bromo-cGMP (+PKG). EJP recordings were taken 1 min before addition of the drug/saline solution and 10 min after the application.
mutant for^{s2} larvae, which express the for^{R} background, respond similarly to neural stimulation as for^{R} larvae but exhibit failure during acute oxidative stress significantly later than for^{R} and for^{s} larvae (Fig. 1, B and C). This suggests that the time to synaptic breakdown during acute H_{2}O_{2} treatment can be manipulated by genetic alteration of PKG pathway function. Further investigations are required to determine the direct mechanism by which PKG signaling promotes protection of neurological function during extreme cellular insults, with a specific emphasis on determining novel molecular interactions, cellular localization(s), and cell expression patterns (Sokolowski and Riedl 1999).

Pharmacological PKG perturbations alter synaptic tolerance to acute oxidative stress. The PKG pathway cannot only be genetically manipulated but also chemically modified via numerous commercially available agents that alter its functional ability by targeting upstream and downstream components. Some examples of such drugs, besides the specific protein kinase effectors used in this study, include T-0156, a potent phosphodiesterase-5 inhibitor (Dawson-Scully et al. 2010; Kikkawa et al. 2001; Mochida et al. 2002); LY83583, a cGMP inhibitor (Schmidt et al. 1985); and Cantharidin, a protein phosphatase 2A inhibitor (Dawson-Scully et al. 2010; Li and Casida 1992). The PKG agonist (8-bromo-cGMP) and antagonists (KT-5823 and Rp-8-bromo-cGMP) used in this study did not solely alter characteristics of the EJP waveform (Fig. 2).

Our pharmacology data further indicate that modifying PKG activity during acute H_{2}O_{2} exposure regulates synaptic transmission in the same manner exhibited by genetic manipulation of this protein kinase. PKG inhibition increased the time until loss of neuromuscular communication was observed, while activation decreased synaptic tolerance to the acute stress by reducing this time frame (Fig. 3).

The modest concentrations of PKG modulators used in this study reinforce the involvement of PKG, opposed to other protein kinases such as PKA or PKC. Studies using cyclic

![Fig. 5. K^{+} channel inhibition protects synaptic function during acute H_{2}O_{2} exposure and PKG pathway modulation. A: decay rate of evoked EJP amplitudes of yw Drosophila larvae during 2.25-mM H_{2}O_{2} treatment (n = 6) is reduced by pharmacologically blocking K^{+} channels. The addition of 250 μM TEA (n = 6) extended the maximum time frame until synaptic failure occurred and this continued in the simultaneous presence of the PKG agonist 40 μM 8-bromo-cGMP (n = 6). B: time to NMJ synaptic transmission failure of yw Drosophila larvae during acute oxidative stress is significantly extended by nonselective K^{+} channel inhibition with TEA solely and in the presence of the PKG activator (Holm-Sidak, df = 2, P < 0.05). Letters in histogram bars represent statistical rankings, where different letters are statistically significant. All vertical bar charts are shown as means ± SE.](http://jn.physiology.org/)

![Fig. 6. Drosophila muscle resting membrane potential and membrane resistance decline during acute oxidative stress is altered by PKG manipulation. A: postsynaptic RMP from yw Drosophila larval muscle 6 was recorded during 2.25-mM H_{2}O_{2} exposure in the following conditions (n = 4 for each group): 2.25 mM H_{2}O_{2} only, 40 μM 8-bromo-cGMP (+PKG), and 50 μM Rp-8-bromo-cGMP (-PKG). B: input resistance from muscle 6 was measured during 2.25 mM H_{2}O_{2} exposure in the following conditions: 2.25 mM H_{2}O_{2} only (n = 4), 40 μM 8-bromo-cGMP (+PKG; n = 5), and 50 μM Rp-8-bromo-cGMP (−PKG; n = 5). Membrane resistance decays at a rate similar to the RMP depolarization and follows the same pattern where PKG activation exacerbates and inhibition reduces the loss of resistance. Inset: representative trace of the voltage response from muscle 6 from 2-nA injected current.](http://jn.physiology.org/)
nucleotide analogs, such as 8-bromo-cGMP, have demonstrated its preference for cGMP-dependent protein kinases compared with cAMP-dependent protein kinases (Francis et al. 1988). Since KT-5823 is known to be not only a potent PKG antagonist (in vitro IC\textsubscript{50} = 234 nM), but also a relatively weak inhibitor of both PKC (K\textsubscript{i} = 4 \mu M) and PKA (K\textsubscript{i} > 10 \mu M) (Hidaka and Kobayashi 1992), we included the more selective inhibitor Rp-8-bromo-cGMP in the study. This metabolically stable analog has been shown to inhibit both PKGI (K\textsubscript{i} = 30 nM) and PKGII and block mammalian cGMP-gated ion channels (IC\textsubscript{50} = 25 \mu M) in excised Xenopus rod photoreceptors (Wei et al. 1996). The observed effects on synaptic transmission during acute oxidative exposure most likely reflect the actions of PKG.

\textit{K}\textsuperscript{+} channel modulation as a potential mechanism for PKG signaling effects. The distinct mechanisms underlying the PKG pathway’s potential for modulating neuronal function and ultimately neuroprotection during acute trauma remain only partially understood. Previous published data indicate a link between \textit{K}\textsuperscript{+} conductance and PKG activity (Chai and Lin 2010; Dawson-Scully et al. 2010; Renger et al. 1999; White et al. 1993), which leads to speculation that these ion channels are potential downstream targets of this intracellular signaling mechanism. However, which specific \textit{K}\textsuperscript{+} channel(s) mediate PKG pathway effects is still a matter of debate. Our drug combination data indicate the likely downstream involvement of \textit{K}\textsuperscript{+} channels during PKG manipulation and acute oxidative stress (Fig. 5). This is in agreement with the proposed model of upstream and downstream components involved in the PKG signaling cascade (Zhou et al. 1996).

Furthermore, our data agree with earlier findings that simultaneous pharmacological intervention with different compounds that effect diverse targets within the PKG pathway results with further downstream component dominating the overall effect (Dawson-Scully et al. 2010). We found that exposure to a \textit{K}\textsuperscript{+} channel blocker (TEA) and PKG agonist (8-bromo-cGMP) during acute oxidative stress protects synaptic function by increasing the time to failure (Fig. 5B). These results mimic what Dawson-Scully et al. reported in 2010, except that we employed \textit{K}\textsuperscript{+} channel inhibition rather than activation. These results are not, however, surprising since Renger et al. (1999) demonstrated conclusively that \textit{K}\textsuperscript{+} conductance in the +PKG and −PKG alleles of the \textit{for} gene in \textit{Drosophila} embryos resulted in differing whole cell \textit{K}\textsuperscript{+} conductance, which was reinforced through the use of pharmacological PKG modulators in this system (Renger et al. 1999). Interestingly, the simultaneous application of PKG activator combined with TEA resulted in a lower but not significant synaptic failure time than TEA alone (Fig. 5A). It is possible that TEA and the PKG activator may operate through competing pathways, but we believe this is unlikely due to a number of factors: 1) due to TEA’s disruptive nature in the preparation, very low dosages are used (250 \mu M) likely resulting in a reduced effect, 2) previous work using activators demonstrates the same pattern of the dominant effect of \textit{K}\textsuperscript{+} modulation combined with PKG modulation during anoxic stress (Dawson-Scully et al. 2010), and 3) finally, it was demonstrated conclusively that \textit{K}\textsuperscript{+} channel conductance is modulated not only by PKG expression and activity levels in \textit{Drosophila} embryos but also by pharmacological PKG modulation (Renger et al. 1999). However, since TEA results in global \textit{K}\textsuperscript{+} inhibition, future work using more specific \textit{K}\textsuperscript{+} channel modulators and \textit{Drosophila} \textit{K}\textsuperscript{+} channel mutants will be required to identify the specific type and cellular localization of \textit{K}\textsuperscript{+} channel(s) responsible for modulating PKG’s effect on neurological function during acute oxidative injury.

Interestingly, we also demonstrate that input resistance declines simultaneously with the depolarization of the postsynaptic (muscle 6) cell RMP in a PKG-dependent manner (Fig. 6). PKG activation shows faster onset of depolarization of the postsynaptic RMP than that of PKG inhibition, where no drug is intermediate (Fig. 6A). Further, PKG activation causes the loss of postsynaptic (muscle 6) input resistance sooner than that of PKG inhibition, where again no drug is intermediate (Fig. 6B). These data demonstrate that modulation of the PKG pathway can alter membrane integrity and this may be through the inhibition of ionic conductances such as \textit{K}\textsuperscript{+}. The finding that resting membrane potential depolarizes along with the reduction of input resistance during this oxidative stress suggests that, at least postsynaptically, there is an overwhelming influx of ions such as Ca\textsuperscript{2+} compared with the potential efflux of \textit{K}\textsuperscript{+}, where one would expect a significant hyperpolarization of the RMP over time. This indicates that future work could also examine low doses of Ca\textsuperscript{2+}, Na\textsuperscript{+}, or Cl\textsuperscript{−} channel blockers to determine their protective effects during acute oxidative stress. It is important to consider that these effects of PKG and/or H\textsubscript{2}O\textsubscript{2} on RMP and input resistance may be muscle specific, and it would be of interest in the future to repeat these experiments in neurons.

These data provide a compelling comparison between genetically and chemically manipulated animals and suggest that PKG activity controls synaptic tolerance to acute oxidative stress in a manner similar to previous findings investigating thermotolerance and anoxic tolerance (Dawson-Scully 2007, 2010). In addition to protection from abiotic stress, PKG pathway inhibition has been shown to rescue the occurrence and severity of spreading depression events in the locust metathoracic ganglion (Armstrong et al. 2009). Spreading depression is associated with numerous mammalian central nervous system disorders including migraine, stroke, and acute brain injury. Therefore, the PKG pathway is a relevant neuroprotective mechanism that provides a potential avenue for creating novel strategies to combat a number of physiological stressors and neurological diseases with the added advantages of evolutionary conservation, diverse model system applications, and the availability of both genetics and rapid pharmacological treatment interventions.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).


