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Effects of *Hyperkinetic (Hk)*, a β subunit of *Shaker* voltage-dependent K^+ channels, on the oxidation state of presynaptic nerve terminals

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Abstract

The *Drosophila Hyperkinetic (Hk)* gene encodes a β subunit of *Shaker (Sh)* K^+ channels and shows high sequence homology to aldoketoreductase. *Hk* mutations are known to modify the voltage dependence and kinetics of *Sh* currents, which are also influenced by the oxidative state of the N-terminus region of the *Sh* channel, as demonstrated in heterologous expression experiments in frog oocytes. However, an *in vivo* role of *Hk* in cellular reduction/oxidation (redox) has not been demonstrated. By using a fluorescent indicator of reactive oxygen species (ROS), dihydorhodamine-123 (DHR), we show that the presynaptic nerve terminal of larval motor axons is metabolically active, with more rapid accumulation of ROS in comparison with muscle cells. In *Hk* terminals, DHR fluorescence was greatly enhanced, indicating increased ROS levels. This observation implicates a role of the *Hk* β subunit in redox regulation in presynaptic terminals. This phenomenon was paralleled by the expected effects of the mutations affecting *glutathione S-transferase S1* as well as applying H_2O_2 to wild-type synaptic terminals. Thus, our results also establish DHR as a useful tool for detecting ROS levels in the *Drosophila* neuromuscular junction.

Keywords

dihydrorhodamine-123; redox; ROS; *Drosophila*; neuromuscular junction; glutathione *S*-transferase; Hydrogen peroxide

INTRODUCTION

Voltage-gated K^+ channels play crucial roles in regulating membrane potentials of excitable cells. Opening of these channels causes repolarizing current flow across the plasma membrane during neuronal activity, and thus defects in these channels increase action potential duration and frequency. *Drosophila Hyperkinetic (Hk)* and its vertebrate homologs (Kv β 1, Kv β 2, Kv β 3) are the β subunit of voltage-gated K^+ channels (Rettig et al., 1994; Chouinard et al., 1995; Heinemann et al., 1995). These β subunits attach to the cytosolic face of their α subunit counterparts encoded by *Shaker (Sh)* and its vertebrate homologs, Kv1 channel genes (Sewing et al., 1996; Yu et al., 1996; Long et al., 2005). Physical interaction between the two subunits determines activation-inactivation kinetics, voltage sensitivity (Rettig et al., 1994; Chouinard et al., 1995), and surface expression (Shi et al., 1996; Accili et al., 1997) of these inactivating I_A channels. Disruptions of the *Hk* gene in *Drosophila* slows down the kinetics and decreases the voltage sensitivity of *Sh* currents in larval muscles (Wang & Wu, 1996). Altered I_A in *Hk* mutants causes neuronal hyperexcitability resembling that of *Sh* mutants, as reflected by

increased firing frequency in cultured central neurons (Yao & Wu, 1999), enhanced neurotransmission at larval neuromuscular junctions (Stern & Ganetzky, 1989), and vigorous ether-induced leg shaking in adult flies (Kaplan & Trout, 1969).

Both *Drosophila Hk* and vertebrate Kv β polypeptides share sequence homology with the aldo-ketoreductase enzyme superfamily (McCormack & McCormack, 1994; Chouinard et al., 1995) that catalyzes the NADP(H)-dependent reduction of a variety of substrates (Jin & Penning, 2007). A recent *in vitro* study has shown that vertebrate Kv β 2 reduces aldehydes to alcohols (Weng et al., 2006). Interestingly, the kinetics of *Sh* and vertebrate Kv1 channels expressed in *Xenopus* oocytes are modified by oxidative and reductive agents (Ruppersberg et al., 1991; Vega-Saenz de Miera & Ruddy, 1992; Duprat et al., 1995; Ciorba et al. 1997). Furthermore, regulation of Kv1.4 channels by the Kv β subunit depends on the redox states (Rettig et al., 1994; Heinemann et al., 1995). However, whether these β subunits contribute to the *in vivo* regulation of reactive oxygen species (ROS) levels has not been determined. Dysfunction of reductase may increase ROS levels and may alter I_A currents mediated by *Sh* and Kv1 channels.

In this study, we examined the cellular oxidation state in *Drosophila Hk* mutants by using dihydrorhodamine-123 (DHR), a fluorescent probe sensitive to oxidation (Kinsey et al., 1987). DHR is a non-fluorescent and uncharged derivative of a fluorescent dye, rhodamine-123. DHR passively diffuses across plasma membrane and is converted to positively charged rhodamine-123 upon oxidation by ROS in the cytosol (Rothe et al., 1988; Emmendorffer et al., 1990). The charged fluorescent product is subsequently trapped in mitochondria due to their extremely low membrane potential (Johnson et al., 1980; Emaus et al., 1986). Therefore, higher levels of ROS result in stronger fluorescence concentrated in mitochondria. Using DHR, our study uncovered that ROS levels are increased in *Hk* presynaptic terminals, suggesting a functional role of the *Hk* β subunit in the cellular redox regulation.

MATERIALS AND METHODS

Drosophila Stocks

The *Drosophila melanogaster* mutant stocks used included *Hk*¹, *Hk*², *gsts*¹⁰⁶²⁵³, *ry*⁵⁰⁶, and *gsts*¹⁰⁴²²⁷; *ry*⁵⁰⁶. The *gsts*1 mutants were obtained from the Bloomington stock center. Second-site lethal mutations carried in the original *gsts*1 stocks were removed by backcrossing to the *ry*⁵⁰⁶ control line eight or more times. These mutant alleles represent independent isolates, and thus provide a means for controlling possible unidentified second-site effects. The wild-type strain, CS, and *ry*⁵⁰⁶ served as controls for *Hk* and *gsts*1, respectively. All stocks were raised at room temperature on standard medium.

Dihydrorhodamine-123 (DHR) staining

Post-feeding third instar larvae crawling on the wall of food vials were chosen for staining. Dissections were performed in HL3 saline containing (in mM) 70 NaCl, 5 KCl, 20 MgCl₂, 10 NaCHO₃, 5 Trehalose, 115 Sucrose, and 5 HEPES, at pH 7.2 (Stewart et al. 1994). Preparations were stained with 1 μ g/ml DHR in saline containing (in mM) 150 NaCl, 5 KCl, 4 MgCl₂, 0.2 CaCl₂, 10 NaCHO₃, 5 Trehalose, 7.5 Sucrose, 5 HEPES, and 1 DMSO at pH 7.2. Pluronic-F (1 ng/ml) was added to help disperse DHR. Subsequently, the preparation was washed with the same saline to remove external DHR. Fluorescence intensity was measured on an upright microscope (Eclipse E600FN, Nikon, Japan) equipped with a Xenon short arc light source (UXL-75XE, Ushio Inc, Japan). Fluorescence from the larval preparation was collected with a water immersion objective lens (40x, Fluoro, N.A. = 0.80), captured with a CCD camera (SM256, Sci Measure Analytical Systems, Inc., Georgia, USA), and stored and analyzed using

the NEUROCCD-SM256 System computer software (Red Shirt Imaging, New Haven, Connecticut, USA). Fluorescence intensity in presynaptic boutons was measured with a FITC filter from neuromuscular junctions on muscles 1, 2, 9, and 10. Before staining, autofluorescence of the preparation was weakened by exposure to excitation light for 20 seconds. To establish the fluorescent reading following DHR staining, brightest areas of 3×3 pixels ($4 \mu\text{m}^2$) within individual boutons were selected and the readings were adjusted by subtracting that from the surrounding areas, where muscle fluorescence was generally very weak. To determine the kinetics of fluorescence increase in mutants and H_2O_2 -treated preparations ($10 \mu\text{M}$), readings at all time points were normalized to the corresponding values of control boutons. DHR was dissolved at a concentration of 1 mg/ml in ethanol, which had been purged with nitrogen, stored at -20°C , and diluted into the saline prior to the application. DHR and Pluronic-F used in this experiment was purchased from Marker Gene Technologies, Inc. (Eugene, Oregon, USA), and Molecular Probes Inc. (Eugene Oregon, USA), respectively.

RESULTS

We used wandering stage third instar larvae for DHR staining because larval neuromuscular junctions at this stage have been extensively studied physiologically (Jan & Jan, 1976; Ganetzky & Wu, 1982; Kuromi & Kidokoro, 1998; Sigrist et al., 2003; Reiff et al., 2005; Ueda & Wu, 2006) and morphologically (Budnik et al., 1990; Atwood et al., 1993; Keshishian et al., 1993). It is relatively simple to localize the synaptic boutons on identified muscles and recognize the bouton types with distinct physiological properties (Kurdyak et al., 1994). We focused on type Ib boutons on muscles 1 and 2, because these boutons are large and their surface localization allows collection of well-focused images of several boutons in the same focal plane. Staining with DHR led to strong fluorescent puncta in the presynaptic boutons and a mesh-like fluorescence pattern in muscle cells with brighter regions surrounding the muscle nuclei. Rh-123 staining generated a similar pattern of fluorescence in pre-synaptic boutons and post-synaptic muscle cells (data not shown). Continuous oxidation by cytosolic ROS during incubation converts DHR to Rh-123 (Rothe et al., 1988), which is accumulated in mitochondria (Emaus et al., 1986; Johnson et al., 1980). Thus, the fluorescence pattern reflects mitochondria-rich regions in post-synaptic muscle cells and pre-synaptic boutons, which are metabolically active and accumulate ROS in the cytosol. Fluorescent intensity was highly heterogeneous in muscle cells, varying from the dimmest within the nucleus to the brightest in the peri-nucleus regions. Nevertheless, the observed fluorescence was still brighter in the presynaptic boutons than in different regions of the muscle cell (Fig.1A).

During the 45 minute staining period, the fluorescence levels of synaptic boutons increased progressively (Fig.1B). To confirm that the rate of fluorescence increase reflects the cellular ROS level, we simultaneously applied DHR and H_2O_2 ($10 \mu\text{M}$). H_2O_2 is known to penetrate the cell membrane and increase the level of cytosolic ROS that oxidizes DHR, but direct reaction between H_2O_2 and DHR outside of the cell is negligible (Henderson & Chappell, 1993; Royall & Ischiropoulos, 1993). In the presence of H_2O_2 , the rate of fluorescence increase in wild-type CS boutons was indeed faster with a steeper slope of fluorescent increase per min (Fig. 2A, readings are normalized to the corresponding values of the control CS), consistent with the idea that higher ROS levels leads to a faster accumulation of fluorescent rhodamine-123. Significantly, in *Hk* mutant boutons, the rise of fluorescence was faster during incubation, resembling the effects of H_2O_2 (Fig. 2A). This observation indicates that the level of ROS is higher in *Hk* boutons.

The above conclusion was further supported by DHR measurements of the internal ROS level in an established redox mutant. Mutants of *gsts1* are known to be defective in the sigma class glutathione *S*-transferase, which conjugates glutathione to reactive oxygen intermediates (Beall et al., 1992; Singh et al., 2001). Increased levels of ROS reaction products in *gsts1* mutants

have been demonstrated immunochemically (Singh et al., 2001). Indeed, *gsts1* mutant boutons displayed a faster rise of fluorescence compared to the control strain *ry*, similar to the effects of *Hk* mutations and H₂O₂ treatment on CS boutons.

After staining for 45 minutes, we washed out DHR from the bath solution and examined the fluorescence intensity for the following 30 minutes (Fig. 1), beyond which the preparations started to deteriorate. We noticed that the general fluorescence levels were weakened by washing the preparation, but the differential fluorescence readings between mitochondria-rich regions and the surrounding regions were not affected. This indicates that the oxidized DHR trapped in the mitochondria is stable and not affected by wash. Notably, the fluorescent intensity of boutons and muscles kept increasing, albeit at a slower rate, after washing the preparation (Fig. 1, Fig 2). We interpret this to indicate continuous oxidation of residual DHR that was not readily washed out. For instance, DHR may remain in certain intracellular compartments not readily accessible to external wash. It should be noted that the post-wash rates of fluorescent accumulation in *Hk* and *gsts1* mutant and H₂O₂ treated CS boutons were not detectably different from that of the controls CS and *ry*⁵⁰⁶.

In muscle fibres, we were not able to establish clear effects of *Hk* and *gsts1* mutations and H₂O₂ treatment. Throughout DHR incubation and wash, the slope of the fluorescence accumulation over time were not significantly different between experimental and control groups (data not shown). The exact reason for the different mutational effects between presynaptic boutons and postsynaptic muscle fibers is not known. Potential explanations include technical difficulties in muscle fluorescence determination and physiological differences between cell types. It is more difficult to standardize the measurements of fluorescence accumulation in muscle cells because of its heterogeneous patterns of DHR staining (Fig. 1). Furthermore, the rates of fluorescence accumulation in muscle during staining and wash were slower than that in boutons (Fig. 1), raising the possibility of different antioxidating systems for coping with ROS in the two cell types. Finally, differential distribution and functional specialization of *Hk* and GST polypeptides may exist in nerve and muscle tissues.

DISCUSSION

A functional role of the β subunit Kv β and *Hk* in the regulation of Kv1 and *Sh* channels has been well established. Although the sequence homology to aldo-ketoreductase is a common feature among different Kv β and *Hk* varieties (McCormack & McCormack, 1994; Chouinard et al., 1995), the functional significance of this well conserved sequence has not been fully explored. Our results establish DHR as a useful tool for probing the cellular oxidation status, enabling the demonstration of an *in vivo* role for the β subunit in the regulation of the cytosolic redox state in the presynaptic nerve terminals. Apparently, a general increase in neuronal excitability is not sufficient to alter ROS metabolism in presynaptic terminals. Examinations of a different hyperexcitable mutant, *quiver*, that also modulates the *Sh* I_A properties and causes ether-induced leg shaking, did not indicate altered rates of DHR fluorescence accumulation (data not shown). Therefore, it will be important in future rescue experiment to compare the efficiency between WT and modified *Hk* constructs in the putative reductase domain to establish the molecular basis of *Hk* enzyme activity. Appropriate GAL4 and UAS lines may be constructive for this purpose.

It is known that both oxidating and reducing agents exert clear effects on synaptic physiology. Oxidizing agents such as diamide and 5,5'-dithiobis(2-nitrobenzoic acid)(3,3'-6) suppress activity-dependent facilitation of synaptic transmission in the lobster neuromuscular junction (Colton & Colton, 1982) and a form of long-term potentiation in rat hippocampus slices (Gozlan et al., 1994; Bernard et al., 1997). In contrast, reducing agents can cause

hyperexcitability in hippocampal neurons, as indicated by increased synaptic facilitation (Tauck, 1992). In *Drosophila Hk* mutants, repetitive nerve stimulation leads to striking enhancement of activity-dependent facilitation of transmitter release, associated with supernumerary firing of presynaptic motor axons (Stern & Ganetzky, 1989). Although this aberrant repetitive firing could be attributed to β subunit malfunction in modulation of the *Sh* I_A channels, additional mechanisms must be considered as well.

First, direct interacting partners of *Hk* may not be limited to *Sh* I_A channels. *Hk* polypeptide is known to bind *Eag*, another voltage-dependent K^+ channel α subunit. Coexpression of *Eag* with *Hk* subunit greatly enhances *Eag*-mediated K^+ current in *Xenopus* oocytes (Wilson et al., 1998). Furthermore, additional K^+ channels have been shown to be modulated by ROS, including a channel encoded by the *human eag-related gene* (*herg*; Taglialatela et al., 1999), and Ca^{2+} -activated BK channels encoded by *slowpoke* (*slo*; DiChiara & Reinhart, 1997). Since the *Eag* subunit has been shown to interact with several K^+ channel subunits (Zhong & Wu, 1993) including *Sh* (Ganetzky & Wu, 1983), *Hk* may affect the various K^+ currents through modulation of the *Eag* subunit.

Certain polypeptides that modulate downstream targets in the signaling pathway are known to be sensitive to cellular redox states. These include protein tyrosine kinases and phosphatases, phospholipases, and transcription factors (Dröge, 2002). Redox regulation of these proteins may affect a variety of cellular processes, including synaptic plasticity, cell proliferation, and neuronal degeneration. Considering that β subunits are widely expressed in the CNS (Rhodes et al., 1995; 1996), the β subunit polypeptide may contribute to the modulation of behaviors through cytosolic redox regulation. *Hk* and *Kv β* mutants display several characteristic behavioral changes. *Drosophila Hk* mutants show ether-induced leg shaking and hypersensitivity to visual stimuli in an escape reflex (Kaplan & Trout, 1969; Engel & Wu, 1998). *Kv β* knockout mice exhibit cold swim-induced tremors and alterations of learning in a water maze paradigm (Giese et al., 1998; Connor et al., 2005). It will be interesting to determine how direct interaction between *Sh* and *Hk* and indirect effects through *Hk* redox regulation of other targets contribute to different components of the behavior of interest.

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Figure 1. Dihydrorhodamine-123 (DHR) staining of *Drosophila* larval neuromuscular junctions (NMJs)

(A) Time-lapsed pseudo-color fluorescent images of NMJs during 45-min DHR staining and following DHR wash-out. These images were high-pass filtered to remove non-specific diffuse fluorescence over the entire field. White-dash lines in the 1st frame demarcate the contour of cellular regions with bright fluorescence in the following frames. N, muscle nucleus; b, presynaptic boutons. Note that boutons display areas of brightest fluorescence surrounded by areas of low fluorescence. (B) Dynamics of the fluorescent intensity in presynaptic boutons and muscles. Symbols in the plot represent data collected from corresponding areas in (A). Arrows indicate the time points when frames in (A) were taken. The fluorescent readings of

the selected areas were adjusted by subtracting the local minimum (surrounding areas, see Methods).

Figure 2. Effects of *Hk* and *gsts1* mutations and H₂O₂ application on the accumulation of fluorescence intensity in presynaptic boutons

(A) Both *Hk* and H₂O₂-treated CS boutons displayed a faster rise of fluorescence compared to the CS control during the 45-min staining period. After washout of external DHR, the rate of fluorescence accumulation decreased (left panel). One-way ANOVA indicates significant increase of the fluorescence accumulation at 45 min in *Hk*¹, *Hk*², and H₂O₂-treated CS boutons (right panel; $p < 0.01$). (B) Boutons in *gsts1* showed a faster rate of fluorescence increase during staining compared to the *ry* control. After wash, the rate decreased (left panel). Fluorescence accumulation at 45 min was significantly greater in *gsts1* mutant boutons (right panel; student *t*-test, $p < 0.02$). Numbers with and without bracket indicate the number of larvae and NMJs,

respectively. Error bars indicate SEM. All readings were normalized to the control level at the end of 45-min staining period.